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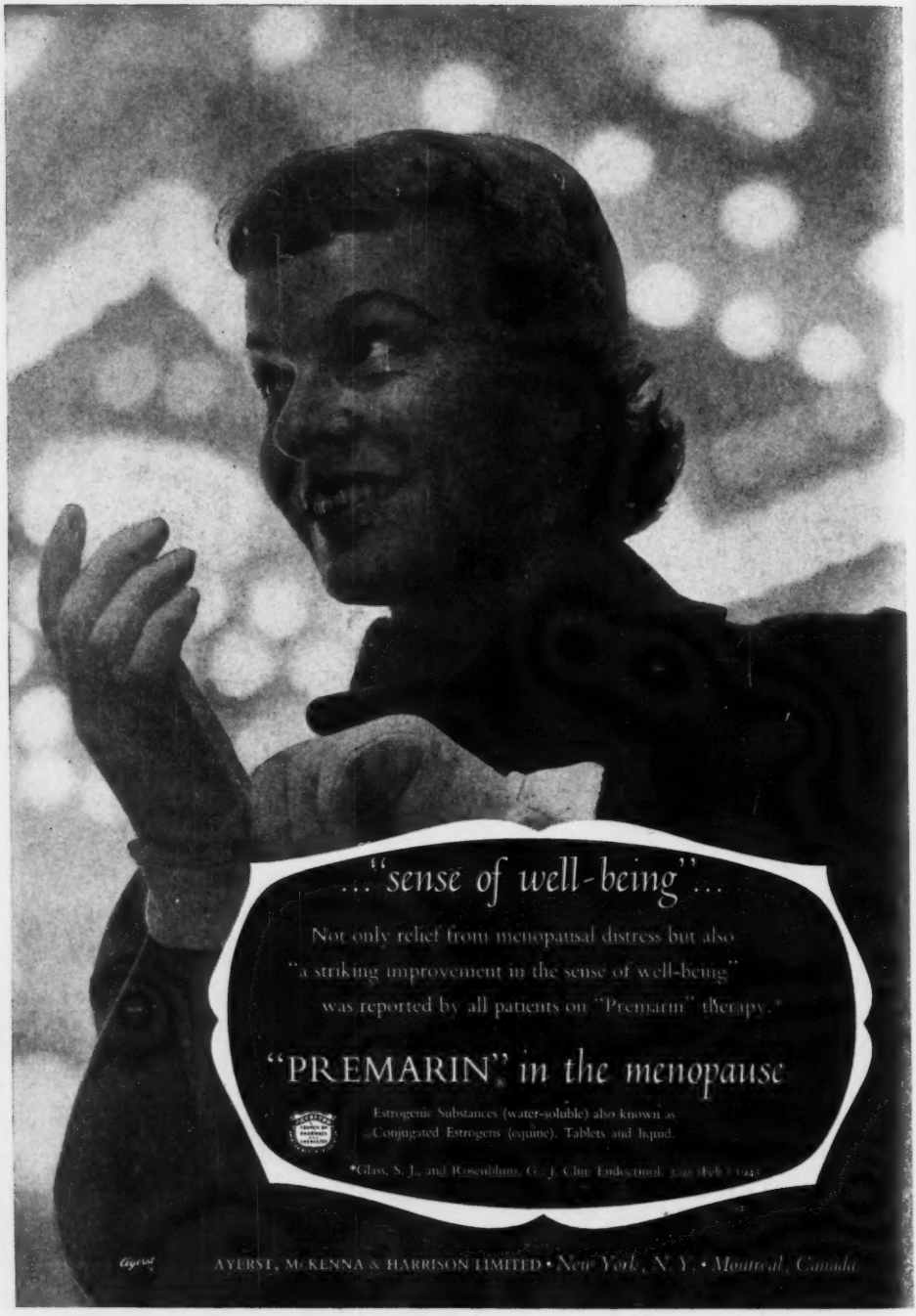
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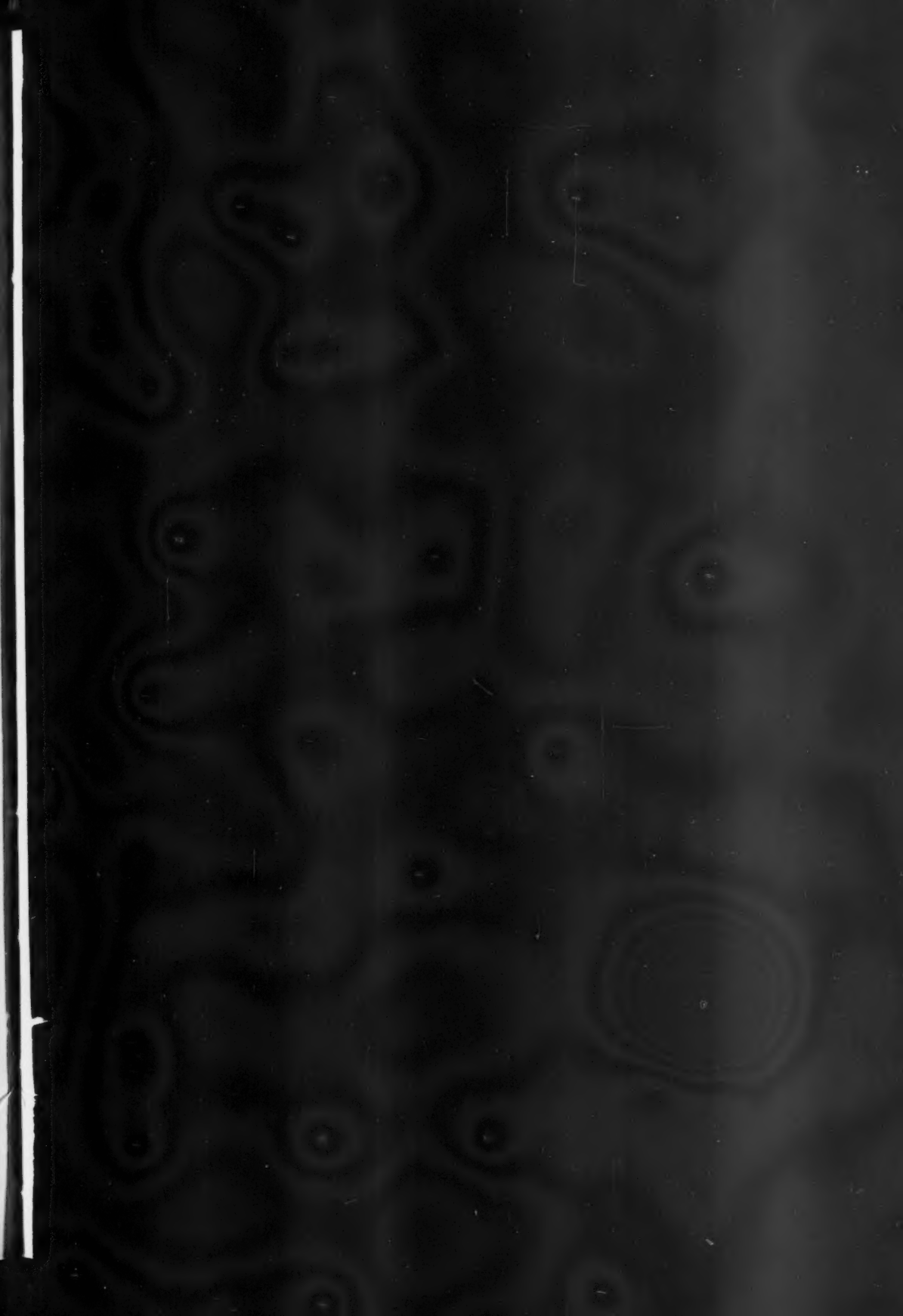

ENDOCRINOLOGY

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CARBOHYDRATE METABOLISM STUDIES ON THE TESTIS OF RATS FED CERTAIN NITROFURANS[1]

HENRY E. PAUL, MARY F. PAUL, FRANK KOPKO, R. C. BENDER AND GEORGE EVERETT

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STRUCTURAL changes of the testes of animals receiving large peroral doses of one of the nitrofurans (Furadroxyl) have been described (Prior and Ferguson, 1950, Nelson, 1953). Similarities were pointed out between the testicular changes seen after nitrofuran administration and those noted in experimental cryptorchidism or after irradiation. Tepperman, Tepperman and Dick (1949) have studied the glucose metabolism *in vitro* of the normal and cryptorchid testis. Lethal doses of X-ray were found to inhibit citrate accumulation in the testis of fluoracetate-treated rats (DuBois, Cochran and Doull, 1951). The sensitivity of the mammalian testis to ionizing radiations has been described by Eschenbrenner and Miller (1950). Pituitary stimulation, as measured by adrenal ascorbic acid lowering in the intact rat, was noted after nitrofuran administration (Paul *et al.*, 1951). However, other unrelated compounds (e.g. acetyl salicylic acid) produced similar adrenal ascorbic acid lowering without testicular changes. These studies and those by Friedgood (1951) on hypophysectomized rats have shown that the nitrofuran action is probably not a secondary effect of pituitary stimulation but is directly upon the testis.

The inhibition of oxygen uptake of normal rat testis *in vitro* by Furacin[2] has been reported (Paul, Paul and Kopko, 1952). Nitrofuran interference with citrate formation by brain and testis from oxalacetate *in vitro* has

Received for publication February 20, 1953.

[1] Presented in part at the Second International Congress of Biochemistry, Paris, July 1952.

[2] 5-Nitro-2-furaldehyde semicarbazone—Brand of nitrofurazone, N.N.R.

recently been shown (Paul, Paul, Kopko and Scanlon, 1952). The present study is concerned with certain phases of carbohydrate metabolism of the testis of control and nitrofuran-fed rats.

METHODS

Oxygen Uptake: Ten albino rats (Supplee Laboratories) were used in the oxygen uptake study. The animals were placed on their respective diets at about 28 days of age and maintained for a period of 145 days. The 5 control animals received a basal diet consisting of GLF[3] calf meal with a multiple vitamin supplement. The experimental animals received the same basal diet with 0.1% added Furacin. The animals were killed by decapitation and the testes removed to beakers containing chilled Krebs-Ringer phosphate buffer. The tissue was prepared by teasing apart the tubules and 100–200 mg. fresh tissue was transferred to each flask. Representative samples of the tissue were used for dry weight determinations. Oxygen consumption was measured by the direct method (Umbreit *et al.*, 1949) in Krebs-Ringer phosphate buffer pH 7.4 in a standard Warburg apparatus. The Q_{O_2} values were calculated for a one-hour incubation period. When glucose was used as a substrate the concentration was 200 mg. per 100 ml.

Citrate Formation: The animals were placed on the respective diets at about six weeks of age and were maintained with food and water *ad libitum* for a two week period. The basal diet was the same as that described in the oxygen uptake experiment and the nitrofurans were incorporated by thorough mixing. The experiment on Furacin was conducted separately with its own controls. The additional two nitrofurans were tested concurrently with a common control. At the end of the two-week feeding period the animals were either sacrificed for the biochemical studies or placed on the basal diet (no added drug) for the indicated periods. Tissue samples for histological study were also taken at the time of sacrifice.

The animals were sacrificed by decapitation. The testis tissue was composited, forced through a 40 mesh stainless steel screen into cold 1.15% KCl; 2 ml. aliquots (about 500 mg. fresh tissue) were transferred by pipet to reaction flasks in duplicate. In the Furacin experiment the incubation medium consisted of 2 ml. of testis suspension, 2 ml. of .01 *M* Krebs-Ringer phosphate buffer pH 7.4 and 1 ml. of potassium oxalacetate solution to give a final .02 *M* substrate concentration. The flasks were shaken in a water bath in air at 37° C. for 60 minutes. In later experiments, to obtain increased citrate formation, the Krebs-Ringer phosphate solution was modified to give a final buffer concentration of 0.1 *M* and anaerobic conditions were used for incubation. The conditions were otherwise comparable. The Dubnoff (1948) apparatus was used in most cases for incubation of the tissue.

Oxalacetic acid was prepared by the method of Heidelberger and Hurlbert (1950). The product was stored in a desiccator in the refrigerator and neutralized with $KHCO_3$ immediately before use. Citrate was determined by the Natelson *et al.* (1948) method. At the end of the incubation period the enzymatic reaction was stopped by the addition of 0.35 ml. of trichloracetic acid solution (100 gm. in 100 ml.) to each flask.

Glucose Utilization: Glucose was measured by the method of Nelson (1944). The tissue was prepared as described above for the citrate determination. A two hour incubation was carried out in air in Krebs-Ringer phosphate buffer in 40 ml. centrifuge tubes suspended in a mechanical shaker in a water bath at 37° C. The initial glucose concentration

[3] Calf meal from Cooperative G.L.F. Mills, Inc., Buffalo, New York.

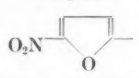
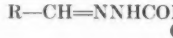
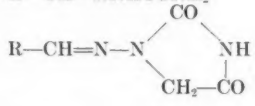
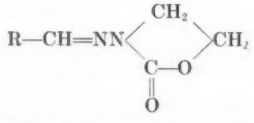
was 0.05% and the volume was 5 ml. The difference in glucose concentrations at the start and at the end of incubation was used as a measure of glucose utilization by the tissue. Suitable controls were conducted simultaneously in all cases.

RESULTS AND DISCUSSION

The oxygen uptake studies on testis tissue of control animals gave Q_{O_2} values (cu. mm. oxygen per mg. dry weight tissue per hour) of 4.7 in the presence of glucose and 2.8 with no added substrate (endogenous). These values are essentially reversed for the Furacin-fed animals, being 3.3 and 4.0 respectively. The average body weight of the animals in both groups was about 300 grams but control testes averaged 3.52 grams in weight whereas the testes of Furacin-fed animals averaged 1.04 grams. Comparison of these oxygen consumption values with those obtained by Tepperman, Tepperman and Dick (1949) on normal and cryptorchid testis shows that the metabolism of the testis of the Furacin-fed animals resembles that of the testis of the cryptorchid animal in that the endogenous metabolism is higher than that of the control and added glucose does not cause increase of Q_{O_2}. The weights of the testes of the nitrofuran-fed animals were approximately one-third of those of the controls. A similar weight relationship was observed by Tepperman *et al.* (1949) for cryptorchid and control testes. Since these preliminary studies indicated a defect in glucose metabolism by the testis of the nitrofuran-fed rat, additional studies on specific phases of carbohydrate breakdown were carried out.

Three nitrofurans differing in side chain structure as indicated in Table 1 were chosen for the study of citrate formation and glucose utilization by testis tissue. Preliminary feeding experiments showed that marked testicular atrophy (weight basis) occurred in animals receiving these com-

TABLE 1. STRUCTURE AND LEVELS OF NITROFURANS STUDIED

Nitrofuran	R equals 	Level used in diet, per cent
Furacin		0.10
Furadantin*		0.25
NF-180 N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone		0.10

* Trade-mark for N-(5-nitro-2-furfurylidene)-1-aminohydantoin.

pounds for two weeks at the indicated levels. Such dosage levels are far higher than those used therapeutically.

The effect of Furacin administration on citrate formation and glucose utilization by testis tissue is shown in Table 2. Examination of these data reveals a marked decrease in the ability of the testis of the Furacin-fed animal to form citrate from oxalacetate while still receiving the nitrofuran or after one day's return to the basal diet. Yet this ability of testis tissue to form citrate has been completely restored one week after cessation of drug administration. On the basis of previous urinary excretion and tissue incubation studies it is known that one day on the basal diet allows removal of drug from the tissues.

Separate studies using glucose as a substrate (Table 2) show that the ability of the testis of the Furacin-fed rat to utilize glucose is also markedly reduced. In contrast to citrate formation, this ability is not regained until testis regeneration is complete, as evidenced by weight and histological appearance. Since glucose utilization in the animals following six weeks regeneration on the basal diet was still not equal to that of the control animals, an additional group was allowed eleven weeks regeneration. Glucose utilization (8.7 μg./mg./hour) was normal at that time and identical to that of paired controls. The testis weight shows a gradual return towards normal during the six weeks regeneration period.

TABLE 2. CITRATE FORMATION AND GLUCOSE UTILIZATION BY TESTIS TISSUE OF CONTROL AND FURACIN-FED RATS AFTER VARYING RECOVERY PERIODS

Group	Recovery period,* days	No. rats	Body weight start, gm.	Body weight end, gm.	Testis weight, gm.	Citrate formation†	Glucose utilization‡
Control	0	4	101	164	2.35	10.8	—
Furacin*	0	4	101	132	1.00	3.5	—
Control	1	5	117	163	2.22	9.1	8.7
Furacin	1	6	120	139	0.93	1.9	0.3
Control	7	3	118	174	2.52	9.8	8.4
Furacin	7	3	116	139	0.92	8.5	0.9
Control	14	3	113	203	2.80	10.9	5.7
Furacin	14	3	111	205	1.38	13.2	1.0
Control	28	1	111	247	3.25	8.3	—
Furacin	28	1	101	230	1.9	12.0	—
Control	42	3	106	243	2.75	11.5	8.4
Furacin	42	3	108	236	2.23	9.7	5.0

* All Furacin-fed animals received 0.1% Furacin in the diet for two weeks prior to starting the recovery period on basal diet. Control animals received basal drug-free diet throughout the test.

† Micrograms citrate formed per mg. dry weight tissue per hour from oxalacetate substrate.

‡ Micrograms glucose disappearing per mg. dry weight tissue per hour.

The results of studies with the two other nitrofurans are to be found in Table 3 and are in accord with the pattern observed with Furacin (Table 2). Although from a weight basis the Furadantin testes appear markedly atrophied, the ability to form citrate was not lowered to the same extent as with Furacin or with NF-180.

In Figure 1 are shown photomicrographs representative of testis tissue structure of control animals, of animals receiving Furacin for a two week period, and of animals showing regeneration of testis tissue at varying intervals after cessation of drug administration. The control testis tissue (Fig. 1A) shows normal proportions of spermatogonia, spermatocytes,

TABLE 3. CITRATE FORMATION AND GLUCOSE UTILIZATION BY TESTIS TISSUE OF CONTROL AND NITROFURAN-FED RATS AFTER VARYING RECOVERY PERIODS

Group	Recovery period,* days	No. rats	Body weight start, gm.	Body weight end, gm.	Testis weight, gm.	Citrate formation†	Glucose utilization‡
Control	1	6	82	125	1.8	18.7	8.6
Furadantin*	1	6	82	120	1.0	9.9	0.0
NF-180*	1	6	82	103	0.8	3.7	0.2
Control	7	6	82	141	2.0	20.4	7.8
Furadantin	7	6	82	141	1.2	21.6	0.2
NF-180	7	6	82	131	0.8	19.0	0.0
Control	14	6	82	170	2.4	16.5	8.8
Furadantin	14	6	82	167	1.4	19.6	0.5
NF-180	14	6	82	166	1.2	20.3	1.3
Control	42	6	82	235	2.8	23.7	7.6
Furadantin	42	6	82	241	2.7	26.0	6.8
NF-180	42	6	82	228	2.1	26.0	3.4

* All nitrofuran-fed animals received 0.25% Furadantin or 0.1% NF-180 in the diet for two weeks prior to starting recovery period on basal diet. Control animals received basal drug-free diet throughout the test.

† Micrograms citrate formed per gm. dry weight tissue per hour from oxalacetate.

‡ Micrograms glucose disappearing per mg. dry weight tissue per hour.

spermatids, and spermatozoa. At the end of two weeks of nitrofuran administration (Fig. 1B) spermatogenesis has been halted at an early stage. There is a complete absence of spermatozoa and spermatids. After one week's return to the basal drug-free diet (not shown in Figure) regeneration is evident in all testis tubules of the nitrofuran-fed animals. However, sufficient time has not elapsed for the cells to have progressed beyond the spermatocyte stage. After two weeks on the basal diet (Fig. 1C), spermatogenesis has progressed to the spermatid stage in many tubules. After six-weeks' regeneration (Fig. 1D) the histological picture is essentially normal.

It is to be noted that these drugs were administered at levels which were not markedly toxic as evidenced by body weight data. In various studies (Friedgood and Green 1950; Prior and Ferguson 1950) it has been pointed

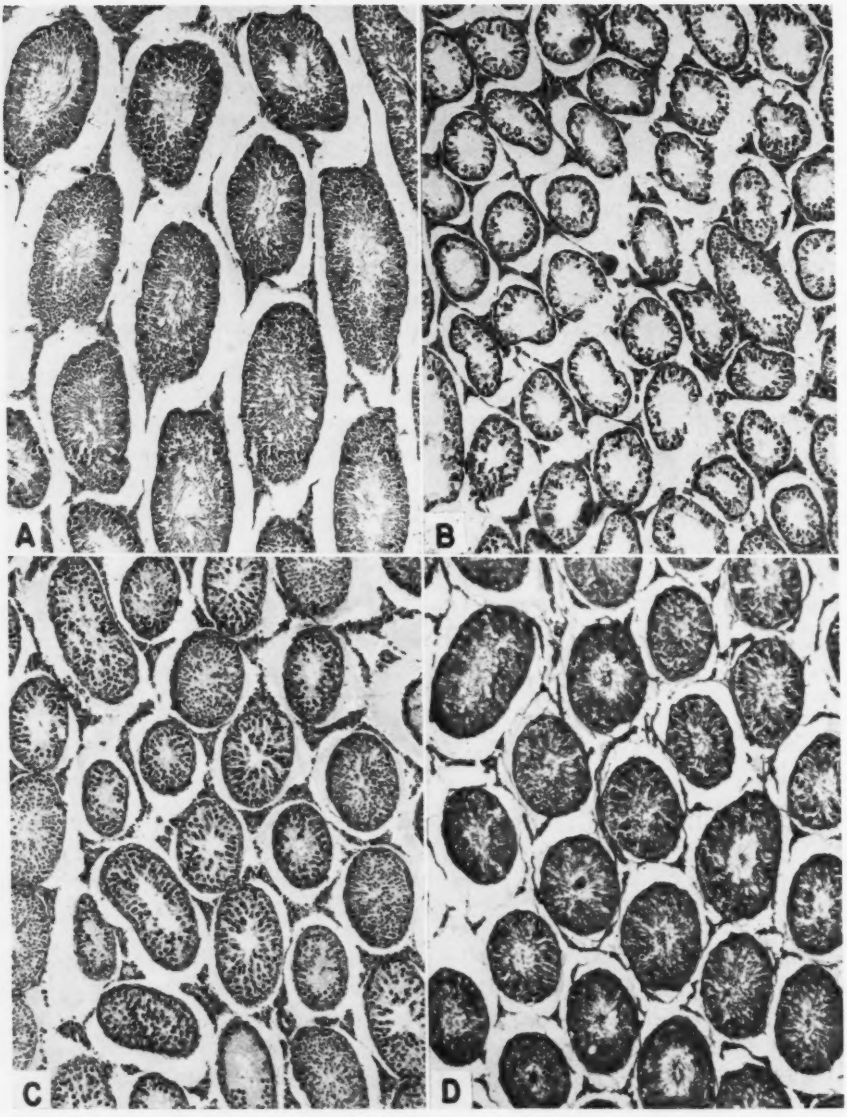


FIG. 1. Cross section of testis of control and of Furacin-fed rats. a. Control rat—Completed spermatogenesis is present in all tubules. b. Furacin 0.1% for two weeks. Spermatogenesis has not progressed beyond the spermatocyte level and in many tubules only spermatogonia and Sertoli cells remain unaffected. c. Furacin 0.1% for two weeks followed by two weeks recovery period. Active regeneration is in progress. Spermatogenesis has progressed to the spermatid level in some tubules. d. Furacin 0.1% for two weeks followed by six weeks recovery period. Spermatogenesis has now progressed to the level of mature spermatozoa. All photographs 80× magnification.

out that chronic administration of the nitrofurans results in little cellular damage other than hypertrophy of the adrenal gland and atrophy of the testes. We conducted an additional experiment in which Furacin was administered to rats on a purified diet at 0.05% level for four weeks. No effect on body growth was observed but testicular atrophy was marked. The ability of the testis tissue of these rats to form citrate was impaired to about the same extent as that reported in Table 2. Determinations with brain tissue from the animals on this 0.05% level showed citrate synthesis equal to that of control animals. Apparently the metabolic reactions of the testis are more sensitive to nitrofuran action than are those of other body tissues.

As noted by Prior and Ferguson (1950) using another nitrofuran, the Sertoli cells and interstitial cells at the end of nitrofuran administration appear essentially normal. Additional evidence that the germinal elements of the testis are most sensitive to nitrofuran action was obtained from organ weight data on animals fed 0.1% Furacin for two weeks. No atrophy of liver, kidney, spleen, heart, thymus, or accessory sex organs was noted.

It seems plausible that during inhibition by nitrofurans, as measured by citrate formation, the germinal elements have been deprived of a nutritive source necessary for cell multiplication. Those elements (Sertoli cells, spermatogonia, etc.) which are apparently dependent on substrates other than carbohydrate for their metabolism (as shown by Tepperman and Tepperman (1950) in the cryptorchid testis) remain normal. When the inhibitor is removed (cessation of drug administration), the necessary nutrient again becomes available and cell multiplication proceeds. The histological picture appears to support this view and shows the ready reversibility of the nitrofuran effect on testicular structure. Complete recovery (including fertile matings) from nitrofuran effects on the testis has recently been reported by Nelson and Steinberger (1953). Biochemical studies (Paul, Paul, and Kopko, 1952b) of the enzyme reactions involved in citrate formation have been carried out to determine more closely the point of interference of Furacin. These will form the basis of a separate communication.

SUMMARY

Oxygen uptake studies indicate a defect in carbohydrate metabolism of the testis of the Furacin-fed rat under well-defined experimental conditions. This defect resembles that shown by other workers for the cryptorchid testis. The Q_{O_2} of normal testis is low in the absence of added substrate and is increased in the presence of glucose. The Q_{O_2} of the testis of the Furacin-fed animal is higher than normal in the absence of added substrate and lower in the presence of added glucose.

The ability of the testis to form citrate from oxalacetate *in vitro* is markedly impaired by the feeding of certain nitrofurans; however, this biochemical defect has disappeared after one week on the basal drug-free diet, and citrate formation then takes place at a normal rate. Glucose utilization is temporarily impaired in the testis of the nitrofuran-fed rat; this effect is also reversible.

From concurrent microscopic studies the effect of three nitrofurans on the biochemical activity of testis tissue appears to be quite closely correlated with observed changes in cellular pattern of testis tubules. The disappearance and return of citrate synthesis is coupled with a corresponding loss and regeneration of specific germinal elements. The ability to utilize glucose returns to normal when tissue recovery has progressed nearly to completion as evidenced by the presence of mature sperm.

Acknowledgment

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SOURCES OF RELAXIN IN THE RABBIT[1,2]

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THE ovary, uterus and placenta have been suggested as possible sites for the formation of relaxin (Herrick, 1928; Hisaw, 1926, 1929; Hisaw, Zarrow, Money, Talmage and Abramovitz, 1944; Zarrow, 1947, 1948). Not only has it been possible to extract relaxin from these three organs but there is some indication that they may serve as sources of the hormone in the normal economy of the organism. However, this work was carried out on different species so that it is difficult to evaluate the relative role of the various sites in the formation of relaxin in a given species. In the sow, the ovary would appear to be the major source (Hisaw and Zarrow, 1948), whereas the placenta may play a more significant role in the mouse (Newton and Beck, 1939; Hall and Newton, 1947) and the guinea pig (Herrick, 1927; Zarrow, 1948).

In view of the many possible sources of relaxin noted among different species, this study was undertaken to determine the major source of the hormone in a single species, the rabbit. This animal was selected because it shows a high titre of relaxin in the blood during pregnancy (Marder and Money, 1944). The studies involve both pregnancy and pseudopregnancy and attempt to evaluate the part played by the placenta, ovary, uterus and vagina in the formation of relaxin.

MATERIAL AND METHODS

All studies for relaxin concentrations were carried out on the tissues of the grey, chinchilla rabbit. The animals were housed under standard conditions at 74° F. and food and water were supplied *ad libitum*. In addition the rabbits received lettuce thrice weekly. Blood was obtained from the marginal vein of the ear or by cardiac puncture, permitted to clot, and the serum removed after centrifugation and stored in the deep freeze. All operations for removal of ovaries, uteri and placentae were carried out under nembutal anesthesia.

The serum was assayed directly for relaxin content while the ovaries, placentae, uteri and vaginas were ground up and extracted twice with 3% HCl. The extracts were kept in the cold at a pH of approximately 0.5 and prior to injection were neutralized with 10% NaOH (Zarrow, 1947).

Received for publication April 22, 1953.

[1] This work was started at the Biological Laboratories, Harvard University, Cambridge, Massachusetts.

[2] Presented at the Federation Meetings, Cleveland, 1951.

The assays for relaxin content were carried out according to the method of Abramowitz *et al.* (1944), except that the guinea pigs were pretreated with 0.5 μg. of estradiol daily for 4 days. Each sample was tested at several dilutions in order to obtain a 67% response which is defined as one guinea pig unit (G.P.U.).

EXPERIMENTAL

Pregnancy and Pseudopregnancy

The initial experiments were designed to determine the relative role of the ovary and placenta in maintaining the relaxin level of the blood during pregnancy. Six rabbits were castrated on the 14th or 15th day of pregnancy and injected daily with 4 mg. of progesterone in two divided doses. The progesterone treatment was continued until the 32nd day after mating and a laparotomy was performed on the 33rd or 34th day.

Fig. 1 is a typical example of the effect of castration on the relaxin content of the blood. It is obvious that the removal of the ovaries did not

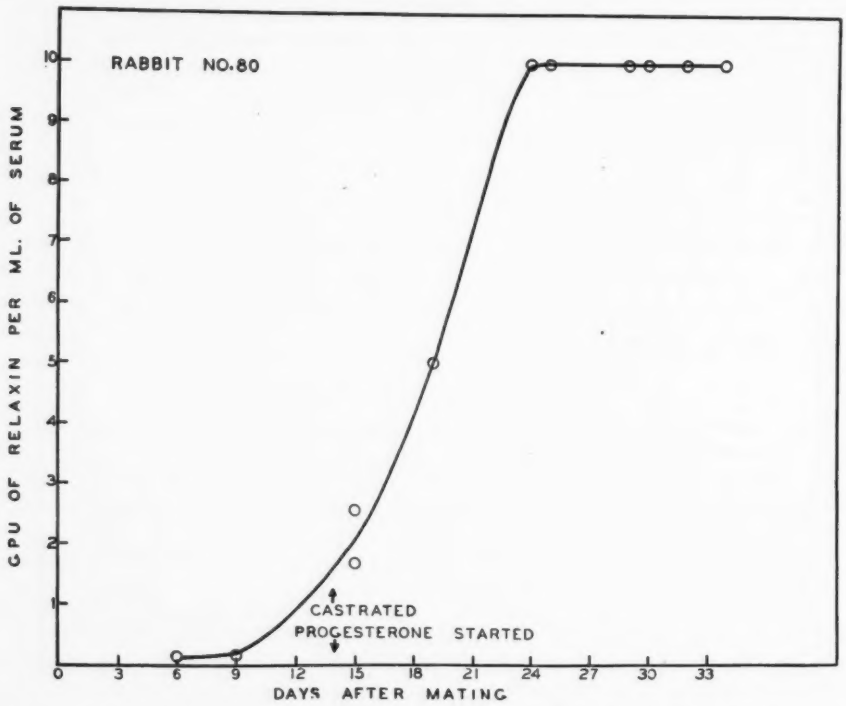

FIG. 1. Concentration of relaxin in a pregnant rabbit castrated on the 14th day and maintained with 4 mg. of progesterone daily until the 32nd day. Postmortem on the 33rd day revealed 8 placentae and 2 dead fetuses.

effect the level of relaxin in the blood. The rabbits showed a concentration of 2 G.P. units per ml. of serum on the 15th day of pregnancy. The concentration then increased rapidly to a maximum of 10 G.P.U. per ml. by the 24th day and was maintained until the animals were sacrificed on the 34th day. It is interesting to note that this treatment failed to maintain completely normal pregnancies in any of the rabbits. In only 3 of the 6 rabbits were any viable fetuses found and in each instance the number was much less than had been observed at the time of castration. However, in all instances the number of placentae was the same as the number of implantation sites seen during castration.

Rabbit No. 85 was castrated on the 14th day of pregnancy and injected with 4 mg. of progesterone daily throughout the experiment (Fig. 2). The relaxin content of the blood followed the normal pregnancy curve until the 32nd day after mating or 18 days after castration. By the 36th day a drop to 7.5 G.P. units of relaxin per ml. of serum was observed and this was followed by a further drop to 5 G.P.U. per ml. by the 41st day. Proges-

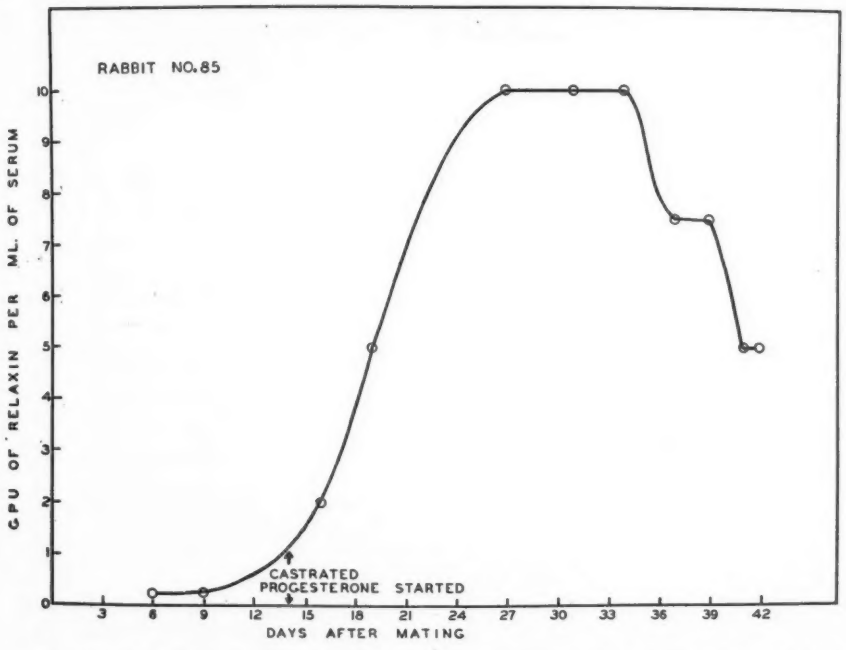

FIG. 2. Relaxin concentration in the blood of a pregnant rabbit castrated on the 14th day of pregnancy and maintained on 4 mg. of progesterone daily until the 39th day. Postmortem on the 42nd day revealed two dead fetuses.

terone treatment was then stopped and a postmortem performed on the 42nd day. Of a total of 14 fetuses seen at castration only 2 dead fetuses remained on the 42nd day. Apparently death had occurred only recently for the fetuses showed increased hair and body growth in comparison with those seen at normal term. The remaining fetuses and placentae were completely resorbed or in advanced stages of resorption.

Analyses of the reproductive tract of the intact rabbit for relaxin revealed a concentration of 30 G.P.U. of relaxin per gram of fresh tissue for the ovary and 50 G.P.U. for the uterus on the 13th day of pregnancy (Table 1). By the 24th day of pregnancy the relaxin of the blood had increased to 10 G.P.U. per ml. but the concentration in the ovary and uterus remained unchanged. However, the placenta was now seen to contain 75

TABLE 1. RELAXIN CONTENT OF THE BLOOD SERUM AND TISSUE OF THE REPRODUCTIVE TRACT OF THE RABBIT

Treatment	No. of rabbits	Relaxin concentration—G.P.U.					
		Per ml. serum	Per gram fresh tissue				
			Ovary	Uterus	Placenta whole	Placenta fetal	Placenta maternal
Pseudo-pregnant	3	0.2 to 0.3	5	50			
Chorionic gonadotropin	4	0.2	5	50			
Pregnant 13 days	3	1.0	30	50			
Pregnant 24 days	2	10.0	25	50	75	10	250
Pregnant 25 days	2	10.0	20	30	50	20	350
Pregnant 26 days	2	10.0	25	15	50	25	200
Pregnant 28 days	1	10.0	25		75		

G.P.U. per gram equivalent of fresh tissue. Separation of the placenta into its fetal and maternal parts revealed an extremely high titre for the maternal placenta (250 G.P.U. per gram equivalent of fresh tissue) as compared with the fetal placenta (10 G.P.U. per gram equivalent of fresh tissue). No further changes were noted on the 25th, 26th and 28th day of pregnancy although a drop was observed in the relaxin content of the uterus.

Several rabbits were made pseudopregnant by electrical stimulation of the uterine cervix during estrus or by the intravenous injection of 150 I.U. of chorionic gonadotropin. After pseudopregnancy was well established the rabbits were exsanguinated and the blood, ovaries and uteri assayed for relaxin. The blood of these animals contained 0.2 to 0.3 G.P.U. of relaxin per ml. of serum, the ovaries contained 5 G.P.U. of relaxin per gram equivalent of fresh tissue and the uterus 50 G.P.U. per gram equivalent of fresh tissue (Table 1).

Castrated, Hysterectomized Rabbits

Five adult rabbits were castrated and hysterectomized and allowed two weeks for post operative recovery. The animals were then injected daily for 30 days with 50 μg. of estradiol. On the 31st day the animals were exsanguinated and the vaginas dissected out and extracted for relaxin. Assays for relaxin content on the blood serum and vagina indicated approximately 0.2 G.P.U. per ml. of serum and 5 G.P.U. per gram equivalent of fresh vaginal tissue.

DISCUSSION

Though a number of investigators have reported the successful maintenance of pregnancy with 3 to 4 mg. of progesterone (Allen and Heckel, 1939; Robson and Sharaf, 1951) the same treatment proved inadequate in the present study in that only 1 or 2 fetuses could be maintained in a few instances. Nevertheless for the purposes of this experiment the treatment produced very interesting results. The ability of the castrated, pregnant rabbit under treatment with progesterone, to maintain a concentration of 10 G.P.U. of relaxin per ml. of serum points to a placental source for this hormone during gestation. This is in contradiction to the pregnant sow where the ovary is the principle source of relaxin (Hisaw and Zarrow, 1948).

The placenta of the normal pregnant rabbit showed a concentration of 50–75 G.P.U. of relaxin per gram equivalent of fresh tissue while the uterus contained 15–50 G.P.U. per gm. However, separation of the maternal and fetal parts of the placenta indicated a 10 to 35-fold difference in concentration between the two, with the fetal placenta containing 10 to 25 G.P.U. per gram equivalent of fresh tissue and the maternal placenta containing 250 to 350 G.P.U. per gm. of fresh tissue. Further evidence for the role of the placenta may be seen in the data from rabbit No. 85 which indicated a relationship between relaxin concentration in the blood and the amount of placental tissue. The above evidence plus the precipitous loss of relaxin from the blood at parturition (Marder and Money, 1946; Zarrow, 1947) points to the placenta as the chief source of relaxin in the rabbit.

Previous work has indicated that the uterus might be considered a source of the hormone (Hisaw *et al.* 1944; Zarrow, 1948). During pseudopregnancy a small amount of relaxin appears in the blood and assays on the ovaries and uteri indicate 5 G.P.U. per gram of fresh tissue for the former, and 50 G.P.U. for the latter.

Finally there is some indication that relaxin may also be formed in the vagina. Experiments involving castrated, hysterectomized rabbits indi-

cate such a possibility. Thus it would appear that the entire reproductive tract i.e. ovaries, uterus and vagina in addition to the placenta can be induced to secrete relaxin.

SUMMARY AND CONCLUSIONS

Pregnant rabbits castrated on the 14th day, and treated with progesterone showed normal levels of relaxin in the blood. A relaxin concentration of 20–30 G.P.U. per gram equivalent of fresh tissue was obtained for the ovary, 15–50 G.P.U. for the uterus, 10–25 G.P.U. for the fetal placenta and 250–350 G.P.U. for the maternal placenta. In view of the failure of castration to affect the normal pregnancy levels of relaxin in the blood and the relatively high concentration in maternal placenta, it was concluded that the placenta is probably the most significant source of relaxin during pregnancy.

During pseudopregnancy both the ovary and uterus must be regarded as potential sources, though the data would indicate that the uterus is a more significant source. Analysis for relaxin, revealed 5 G.P.U. per gram of ovary and 50 G.P.U. per gram of uterine tissue. Prolonged estrogenic treatment in a rabbit devoid of ovaries and uterus revealed the presence of 0.2 to 0.3 G.P.U. per ml. of serum and 5 G.P.U. per gram of vaginal tissue. It would appear that in the rabbit, under suitable conditions, the ovaries, uterus, placenta and vagina may all secrete relaxin.

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THE STATE OF PROGESTERONE IN PLACENTAL TISSUE[1]

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NOALL *et al.* (1953) recently suggested that in placental tissue "protein interacts with progesterone to form a stable complex from which the progesterone is not easily extracted by organic solvents." This inference was apparently based on the failure by Pearlman and Cerceo (1952a) to isolate any progesterone from 836 kilos of human placental tissue whereas the former authors (1953) obtained approximately 1 mg. of progesterone per kilo of tissue following alkaline digestion and ether extraction. Pearlman and Cerceo (1952b) had likewise isolated progesterone from this source in comparable yield by the latter procedure.

The concept advanced by Noall *et al.* (1953) is not supported by certain preliminary observations, details of which were omitted from our previous reports (Pearlman and Cerceo, 1952a, 1952b) in the interests of brevity. In our subsequent small scale operations, a substantial amount of α, β-unsaturated ketones was obtained by acetone extraction of *fresh* placental tissue despite the failure to isolate progesterone in the large scale operation. In continuing our search for progesterone, the acetone extraction procedure was abandoned in favor of the alkaline digestion-ether extraction primarily because the former was time-consuming and cumbersome. Pertinent details of some of our preliminary observations and certain data subsequently obtained are now presented.

EXPERIMENTAL

A summary of the following data, particularly as it pertains to the yield of progesterone obtained by various extraction procedures, is included in Table 1.

Acetone extraction of placenta.—1.9 kg. of full term human placental tissue were finely ground and extracted repeatedly with acetone at room temperature over a period of several days. The acetone concentrate was extracted with ether; the ether residue was partitioned between petroleum

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[1] This investigation was supported by a research grant (RG 2567 (C and C2)) from the National Institutes of Health, United States Public Health Service.

TABLE 1. A COMPARISON OF THE YIELD OF PROGESTERONE OBTAINED FROM HUMAN PLACENTA BY VARIOUS EXTRACTION PROCEDURES

Extraction procedure	Progesterone content per kilo of tissue	Reference; remarks
	mg.	
A. Alkaline-digestion, ether. (room temperature)	0.93*	Pearlman and Cerceo (1952b); 12.5 kilos extracted; countercurrent distribution of ketones; crystalline progesterone isolated in 60% yield.
	1.0–1.5†	Pearlman and Cerceo (1953); 7 batches of tissue individually extracted.
	approx. 1.0	Noall et al. (1953); 11.2 kilos extracted; value based on isolation of crystalline progesterone and in agreement with bioassay (intrauterine technique).
	2.4*	This study; a 100 gm. aliquot portion of placenta extracted.
B. Acetone (room temperature)	1.8*	This study; another 100 gm. aliquot portion of same placenta (see above) extracted.
	1.5†, 1.3†	This study; batches of 1.9 and 0.44 kilos respectively extracted.
C. Boiling ethanol (procedure of Allen (1932)).	approx. 3	McGinty *et al.* (1936); approx. 10 kilos extracted; progesterone estimate based on bioassay data.‡

* Based on measurement of the optical density of the ketonic fraction at 240 mμ.

† Based on measurement of the optical density at 301 mμ of the thiosemicarbazones derived from the ketonic fraction.

‡ Similar studies (cited by McGinty *et al.* (1936)) have been reported but the bioassay values vary considerably. In the initial studies on placenta, progestational activity failed to be detected owing to contamination of the extracts with estrogenic material, an inhibiting factor in the bioassay.

ether and 70% methanol. The residue from the latter phase was treated with Reagent T (Girard and Sandulesco, 1936) yielding 23 mg. of ketonic material. Its ultraviolet absorption curve was poorly defined; a peak at 240 mμ could not be discerned. However, estimation of progesterone by a micro thiosemicarbazide reaction procedure (Pearlman and Cerceo, 1953) indicated a content of 2.8 mg. of progesterone; well-defined maxima in the ultraviolet absorption curve of the thiosemicarbazones were observed at 279 and 301 mμ.

The acetone-defatted placental tissue was subjected to continuous extraction with methanol in a percolator for 16 hours. The extract was worked up in a fashion similar to that described above. The ketonic fraction was treated once more with Girard's Reagent T and the final ketonic fraction partitioned between petroleum ether and 70% methanol. The residue (21 mg.) from the latter phase gave a poorly defined ultraviolet absorption curve. The thiosemicarbazones were prepared; these gave an

ultraviolet absorption curve which was well defined, exhibiting a single peak at 273 mμ, an indication of the presence of saturated ketones; there was no spectroscopic indication of progesterone bisthiosemicarbazone. It was estimated that approximately 26 mg. of saturated ketonic material were present, expressed as mg. equivalents of allopregnanolone (solely as a reference standard).

Another placental specimen (0.44 kg.) was similarly extracted with acetone; the progesterone content was estimated to be 0.55 mg. according to the thiosemicarbazide reaction procedure. A methanol extract of the acetone-defatted tissue was not made in this case.

Acetone extraction versus alkaline digestion-ether extraction.[2]—100 gm. of placental tissue were extracted with acetone by the method described above. The ketonic fraction was dissolved in 0.4 ml. of dry ether and passed through a narrow column containing 300 mg. of alumina[3] (Harshaw) previously wetted with the same solvent; elution was made with 20 ml. of dry ether. The eluate (in ethanol) gave an ultraviolet absorption curve typical for an α, β-unsaturated ketone (maximal absorption at 240 mμ). The progesterone content was estimated to be 0.178 mg. Chromatography of 0.250 mg. of crystalline progesterone under identical conditions gave an 84 per cent recovery of the hormone, determined spectroscopically.

From the same placental specimen, 100 gm. of tissue were worked up by an alkaline digestion-ether extraction procedure (Pearlman and Cerceo, 1952b). The ketonic fraction was chromatographed, furnishing an eluate which gave a well defined ultraviolet absorption curve of the type mentioned above. The progesterone content was estimated to be 0.236 mg.

DISCUSSION

The ketonic material obtained from acetone extracts of placental tissue is not suitable for ultraviolet spectroscopy nor is the extraction of lipides complete. That this material contains α, β-unsaturated ketones in substantial amounts is evident from ultraviolet spectroscopic examination of its thiosemicarbazones. In the experiments described in this report, 1.5 mg. and 1.3 mg. of progesterone per kilo of tissue were thus estimated to be present. These values fall within the range of 1.0 to 1.5 mg. of the hormone per kilo of tissue recently reported by the authors (Pearlman and Cerceo, 1953) following alkaline digestion-ether extraction and application of the thiosemicarbazide reagent.

[2] The following set of data were presented before the meeting of the Endocrine Society at Chicago, 1952 but were not included in the abstract (Pearlman and Cereco, 1952c).

[3] The preparation is described in footnote 4 (Pearlman and Cerceo, 1952a).

Our experiments demonstrate that interfering material can be readily removed from the ketonic fraction of acetone extracts by simple chromatography to permit direct spectroscopic examination of the eluate for progesterone; some loss in hormone recovery can be expected by this procedure, however. When aliquot portions of a placental specimen were examined, 1.8 and 2.4 mg. of progesterone per kilo of tissue were found in the acetone and alkaline digestion-ether extracts respectively.

That the α, β-unsaturated ketones in both types[4] of extracts contain a major proportion of progesterone[5] was demonstrated by Diczfalusy (1952) who described an elegant method for the estimation of progesterone in placental tissue by countercurrent distribution and ultraviolet spectrophotometry. Determinations were not made on aliquot portions of the placental specimens, however, and a direct comparison of yield of hormone by the two extraction procedures was not drawn. Diczfalusy (1952) considered the intriguing possibility of "protein-bound" progesterone but reserved comment for a future communication.

A comparison of the yield of progesterone obtained from human placenta by various extraction procedures is presented in Table I. The authors are still at a loss to explain the failure to isolate progesterone by acetone extraction in our initial endeavor (Pearlman and Cerceo, 1952a); closely related C_{21} steroids were nevertheless obtained. Perhaps the freshness of the placenta is a critical factor as appears to be the case with certain other endocrine tissues, e.g. the adrenal cortex. Although the alkaline digestion procedure has been favored in this laboratory, it has the drawback that steroid hormones of the adrenocortical type will undoubtedly be destroyed.

SUMMARY

The yields of α, β-unsaturated ketones obtained from human placental tissue by acetone extraction and by procedures involving alkaline digestion and ether extraction are of the same order of magnitude. The estimation is based on ultraviolet spectroscopy.

These findings militate against the view recently advanced that protein interacts with progesterone in placental tissue to form a stable complex from which the progesterone is not easily extracted by organic solvents. Apart from this consideration, our data do not argue for or against the existence of a progesterone-protein complex.

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[4] The classic Allen (1932) extraction procedure was employed by Diczfalusy (1953); however, the acetone-methanol extraction procedure described above is essentially the same.

[5] Or more properly, that of an α, β-unsaturated ketone with partition properties identical with that of progesterone.

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ASSAY OF THE FOLLICLE STIMULATING HORMONE BASED ON THE AUGMENTATION WITH HUMAN CHORIONIC GONADOTROPIN

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INTRODUCTION

THERE have been in the past numerous attempts to quantitatively assay the follicle stimulating hormone (FSH) of the anterior pituitary. Fevold *et al.* (1937, 1940) and many others have used the increase in ovarian weight in immature female rats. Evans *et al.* (1939) have employed an histological assay based upon the production of healthy (non-atretic) follicles with small antra in the ovaries of hypophysectomized female rats. Many other workers use uterine weight as a criterion for the estimation of FSH activity. In hypophysectomized male rats Greep *et al.* (1940, 1942) have employed the increase in testicular weight, in the absence of hypertrophy of the prostate and seminal vesicles, as a means of determination of FSH.

It has been known for some time that human chorionic gonadotropin (HCG) will augment the action of FSH in the ovary. Bates and Schooley (1942) in their excellent article on this subject indicated that it might be possible to use it as an assay method. Their work was confined to the use of a single preparation of unknown FSH and LH content. The degree of contamination with other hormones was not reported.

Simpson, Li and Evans (1951) showed that highly purified FSH when administered with HCG to hypophysectomized female rats gave an augmentation in ovarian weight. It was demonstrated that increases in FSH dosage produced a graded dose response. The levels of FSH used were such that when given alone, they did not show LH activity as evidenced by histological examination for the repair of interstitial tissue. The preparation was not contaminated to any extent with any of the known hormones. Therefore, it is logical to assume that the FSH is responsible for the augmentation response with HCG.

In the present studies it has been demonstrated that fairly high dosage with HCG will make the intact immature female rats very sensitive to exogenous FSH and that within certain dosage ranges, the relationship

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between administered FSH and ovarian weight is linear. It has been further shown that reasonable amounts of other pituitary hormones including LH do not interfere with this response.

ASSAY PROCEDURE

In all experiments twenty-one to twenty-two day old Sprague-Dawley female rats were used. The injection schedule was three one-half milliliter subcutaneous injections per day for three days. All ingredients were mixed together in physiological saline and given as a single injection. Autopsy was on the fourth day (72 hours after the first injection). The ovaries were removed, dissected free of surrounding tissue and weighed on a Roller-Smith torsion balance to the nearest one-tenth of a milligram.

MATERIALS

Follicle Stimulating Hormone Standard.—A swine FSH preparation (264-151-X) made in the Armour Laboratories by the method of Steelman *et al.* (1953) was employed as a standard. Its activity, electrophoretic composition and contamination are given in Table 1. Other samples of FSH were kindly furnished by Dr. C. H. Li and Dr. H. B. van Dyke.

Other Pituitary Hormones.—A sample of sheep LH free of FSH was supplied by Dr. M. E. Simpson. All other pituitary hormones used were prepared in the Armour Laboratories and were relatively free of other contamination hormones, especially gonadotropins.

Human Chorionic Gonadotropin.—Commercial grade human chorionic gonadotropin was employed and contained 750 I.U. per milligram.

RESULTS

Preliminary data established the fact that when 10–50 I.U. of HCG per animal was given, as little as 0.1 to 0.2 mg. of FSH caused a significant increase in ovarian weight over that of the HCG control. When FSH was given in the above amounts without the HCG, there was no significant change in ovarian weight over that of the saline control. Three successive experiments were run, and in each experiment, increasing amounts of FSH were administered concomitantly with 10 I.U., 20 I.U., and 40 I.U. of HCG. Experiment 81 shown in Figure 1 is one of these experiments and will serve as an example of all three. Ninety-five animals were used for Experiment 81 and all but one point on the Figure represents the average ovarian weight of both ovaries of six animals (one point is the average of five animals).

The possibility that one ovary may be more responsive than the other was investigated. The ovaries of all ninety-five animals were weighed separately and the significance of the difference between the right and left ovaries was tested by means of the Student "*t*." No significant difference was found and all further work was done with the combined weight of both ovaries.

TABLE 1. BIOLOGICAL ASSAY DATA OF FSH STANDARD (264-151-X)

Assay	Procedure	Results			
		Total dose	Number of animals	Average ovary weight	Average uterine weight (stripped)
FSH					
Hypophysectomized female rats	SQ: 1 injection per day for 3 days: autopsy at 72 hrs.	Saline 2.0 mg.	6 6	8.2 mg. 38.7	15.4 mg. 70.7
Intact immature female rats	Same as above	0.1 mg. 0.2 gm. 0.3 mg. Saline	5 5 5 5	12.9 mg. 14.8 19.6 12.1	
LH					
Hypophysectomized male rats	SQ: 1 injection per day for 7 days; autopsy on 8th day	Approximately 1% of 227–80*			
Intact immature male rats	SQ: 1 injection per day for 4 days: autopsy on 5th day	<3% of 227–80*			
TSH	Iodine depletion in day-old chicks	<0.04 U.S.P. units per mg.			
ACTH	Adrenal ascorbic acid depletion	<0.01 I.U./mg.			
Prolactin	Pigeon crop sac	<0.25 I.U./mg.			
Growth	Body weight increase	<2% of 22KR2†			
Oxytocin	Chicken blood pressure	<0.02 U.S.P. units per mg.			
Pressor	Cat blood pressure	<0.012 units per mg.			
Adipokinin	Mouse liver fat	Nil			
Exophthalmos Prod. Sub.	Fundulus	Nil			
Electrophoresis	Veronal buffer (pH 7.8)/2 0.05; protein concentration of 1.10%	*Component* 1 2 3 4‡	*Mobility* (*x* 10-5) −1.15 −2.36 −3.62 −5.44	*Percentage* 7 10 12 71	

* Sheep LH-15 Mcg. gave 100% increase in ventral prostate by Greep assay (1940).
† Electrophoretically homogeneous beef growth hormone (Armour Standard).
‡ From data at other pH's, the isoelectric point of this component is pH 4.8. This isoelectric point and mobility are approximately the same as those of van Dyke (1950).

An investigation of the differences in body and ovarian weights in 21–22 day animals was also carried out. It was found that animals of the same age did not show a significant difference in ovarian response despite variation in body weight.

The mean values for each dose of HCG and FSH in Experiment 81 and the least squares regression line for each dose of HCG are shown in Figure 1. The slope of each regression line, b; the standard error of b, s_b; and the ratio $t = b/s_b$ are given below:

HCG Dose	b	s_b	t
40 I.U.	431	47	9.18
20 I.U.	344	29	11.78
10 I.U.	133	21	6.25

The slopes are all significantly different, i.e., the slope for 40 I.U. is significantly greater than for 20 I.U.; and for 20 I.U., greater than for 10 I.U. The standard error of the slopes, s_b, are also significantly different, the larger slope having a significantly higher error.

The mean square or variance of the deviation of the mean ovarian weight at each dose from the value calculated from the regression line (deviations

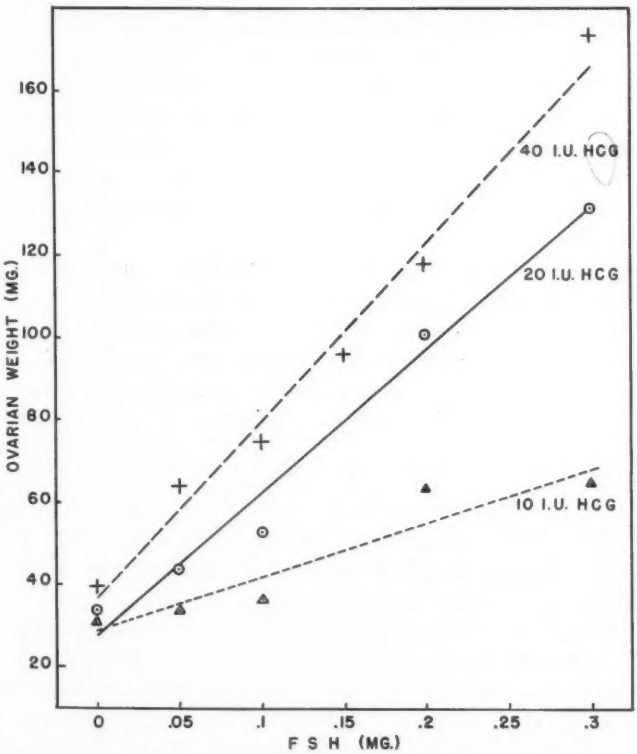

FIG. 1. Regression lines for assays of FSH augmented by 10, 20, and 40 I.U. of HCG.

from regression mean square) was compared to the pooled variance of the individual ovarian weights at each dose (error mean square). For an experiment which has 5 or 6 dose levels, and six animals at each dose, an F ratio (deviation from regression mean square divided by error mean square) of about 3 or more would indicate that the response was not linearly related to the dose. An F ratio of less than 3 shows that the dose-reponse relationship is linear. It may be seen from the data below that all three dosages of HCG gave linear dose-response curves:

HCG dose	Deviations from regression mean square	Error mean square	F ratio
40 I.U.	254.89	838.95	<1
20 I.U.	301.54	288.83	1.04
10 I.U.	243.81	145.76	1.67

No significant differences were found between the deviations from regression mean squares at each of the doses of HCG. The error mean square for the 40 I.U. was significantly larger than the error mean square for either 20 I.U. or 10 I.U., but the latter two were not significantly different. Because of the large error mean square of 40 I.U. and the small b value and small t of 10 I.U., it was decided that 20 I.U. of HCG would be used as the standard augmenting dose.

All work reported in the following sections is based on an augmenting dose of 20 I.U. per animal.

After a set assay procedure was established, it was decided that additional studies should be undertaken. Since the problems of hypophysectomized animals versus intact animals, contamination with other hormones, etc., constantly arise in all biological assay procedures, these various points were investigated. In addition to the above, a section on statistical considerations and calculations is included.

THE EFFECT OF HYPOPHYSECTOMY ON THE AUGMENTATION RESPONSE

Simpson *et al.* (1951) have shown that the ovarian weight response of highly purified FSH preparations can be augmented with HCG in hypophysectomized animals. However, there was no attempt made to quantitate the response for assay purposes.

It was thought that hypophysectomy of the animals would improve the assay since it would eliminate the influence of the animal's own pituitary. Two assays were conducted in which hypophysectomized animals were compared with intact animals. The assays were carried out using the standard 20 I.U. of HCG and the hypophysectomies were performed 24 hours prior to the start of the first injection.

Table 2 summarizes the data and Figure 2 shows graphically the responses for Experiment 92. The hypophysectomy tended to reduce the error mean square and the slope of the response, but the deviations from regression were increased. The latter caused a high F ratio and showed that the response of the hypophysectomized animals was not linear in the dosage range used in the experiment.

Since the intact animals gave better slope and t values, and due to the fact that the hypophysectomized animals did not produce a linear response curve with the doses employed, it was decided to use the intact animals for purposes of assay. It should be pointed out, however, that it is conceivable that hypophysectomized animals could be used if higher HCG and FSH doses were used, or the conditions of the assay changed in some other man-

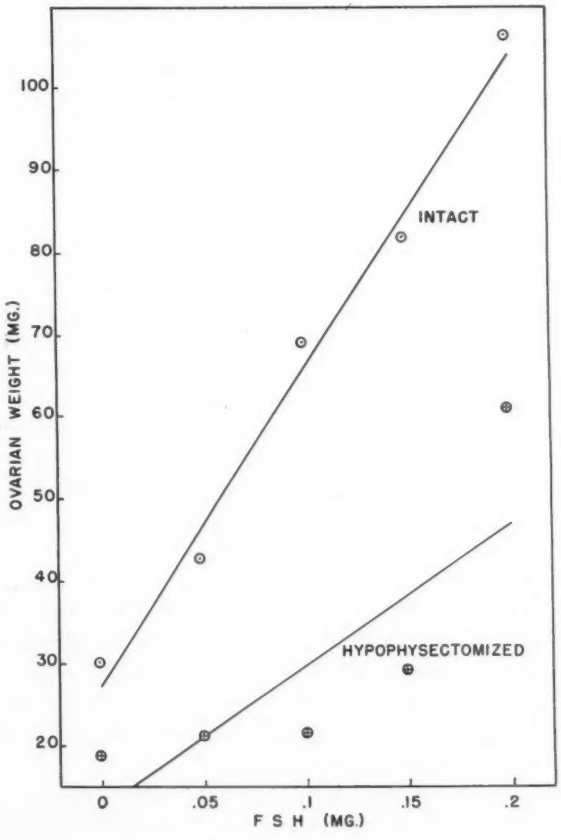

FIG. 2. Regression lines for assay of FSH in intact and hypophysectomized animals.

TABLE 2. COMPARISON OF THE RESPONSES OF INTACT AND HYPOPHYSECTOMIZED ANIMALS TO THE AUGMENTATION OF FSH WITH HCG

	Experiment 92		Experiment 93	
	Hypophysectomized	Intact	Hypophysectomized	Intact
Number of animals	29	40	34	40
b	172.39	382.08	193.48	368.97
s_b	35.91	42.50	20.09	36.83
$t=b/s_b$	4.80	8.99	9.63	10.02
Deviation from regression	670.96	151.29	386.85	287.37
Error mean square	119.24	379.32	92.81	550.78
F ratio	5.63	0.40	4.17	0.52

ner. Higher FSH doses would result in the additional complication of correcting for the ovarian weight due to the FSH alone. When given without HCG, doses of FSH greater than 0.2–0.25 mg. produce ovarian weights significantly greater than those of the saline controls. Results with the intact animals have been such that it was not felt that further investigation of hypophysectomized animals was justified.

THE EFFECT OF OTHER PITUITARY HORMONES ON THE AUGMENTATION RESPONSES

Five anterior pituitary hormones were tested for their action on the augmentation response. In each case a dose of the standard FSH was "spiked" with the contaminating hormone. Ovarian responses were then calculated against the standard which was run in the conventional manner. In addition, a sample of the added hormone, equivalent in weight to that administered on the highest dose, was given with the HCG alone. In no case was there a significant difference between the HCG control and the HCG plus the added hormone. The method of calculation given in a subsequent section was used in all cases. Dosage levels of 0.1 and 0.2 mg. of FSH were used in all instances except where there were only eight animals, in which case, a dose of 0.1 mg. was employed. All levels of standard and standard plus other hormone contained eight animals. A summary of the data is given in Table 3.

Two sheep LH preparations were tested for interference with the FSH response. One was a preparation furnished by Dr. Miriam E. Simpson and said to be almost devoid of FSH activity. The other was an LH sample prepared by one of the authors and shown to be relatively free of FSH but having excellent LH activity as measured by the ventral prostate response in the hypophysectomized male rat.

It is interesting that both LH samples in fairly large amounts did not interfere with the augmentation response. Since HCG is an LH-like substance, and the amounts given under the conditions of assay are quite large, the ovaries were evidently overwhelmed and the addition of exoge-

TABLE 3. THE EFFECT OF VARIOUS PITUITARY HORMONES ON THE AUGMENTATION OF FSH WITH HUMAN CHORIONIC GONADOTROPIN

Hormone contaminant	Per cent of FSH dose	Number of animals	Assay value of FSH & hormone (% 264-151-X)
Sheep LH SXIV 52*	50	16	106 ± 16%
Sheep LH† (227-80)	5	16	111 ± 14
	5	16	89 ± 14
	10	16	112 ± 19
	10	8	90 ± 12
	25	16	90 ± 12
	50	16	83 ± 11
Beef TSH (317-51) 1.3 USP μ/mg.	25	16	116 ± 20
	25	16	81 ± 12
	50	16	128 ± 19
	50	8	93 ± 15
Beef Prolactin 13-15 I.U./mg.	25	16	115 ± 19
	50	8	85 ± 17
Hog ACTH‡	250	16	86 ± 12
	500	16	69 ± 28
Beef growth hormone§ (22KR2)	25	16	112 ± 12
	50	16	101 ± 12

* Kindly furnished by Dr. M. E. Simpson of the University of California. This preparation was found to be of comparable potency to 227-80 when assayed by the ventral prostate method in hypophysectomized male rats.

† Preparation 227-80 at a total dose of 15 micrograms increased the ventral prostate weight by 100% in an hypophysectomized male rat in four days.

‡ Expressed in terms of LAIA (1 I.U./mg.)

§ Essentially homogeneous by electrophoresis.

nous pituitary LH did not produce any exaggerated response. If anything, the pituitary LH at high dose levels (in the case of preparation 227–80) seemed to depress the response although the depression was not significant at the 95% confidence level.

THE ASSAY OF OTHER FSH PREPARATIONS

Through the courtesy of Dr. C. H. Li and Dr. H. B. van Dyke, samples of FSH prepared in their laboratories were assayed for comparison purposes. Both of these preparations gave satisfactory dose response curves. They were assayed for LH in hypophysectomized male rats and found to be largely free of this hormone. The assay data for two of Dr. Li's samples and one of Dr. van Dyke's are given in Table 4.

STATISTICAL CONSIDERATIONS

It has been previously shown that the relationship between ovarian weight and FSH is linear. A study of the data indicated that the response is not a log-dose relationship. However, if the HCG control value is ig-

TABLE 4. THE ASSAY OF FSH PREPARATIONS FROM OTHER LABORATORIES

Sample	Species	Number of assay levels	Potency (% of 264-151 X)
Li	Sheep	2	134 ± 21
		2	137 ± 23
Li (11631)	Sheep	2	87 ± 14
		2	99 ± 15
Van Dyke (446-CC)	Hog	2	69 ± 12
		2	52 ± 12

nored and only responses falling between 0.1 and 0.2 mg. of standard FSH are used the log-dose method of Irwin (1937) gives similar results to those obtained by the method proposed. For samples whose ovarian responses fall between the HCG control and the 0.1 mg. dose level the log-dose method gives erroneous results as would be predicted from the statistical analysis.

The method of calculation proposed is the slope-ratio method of Finney (1950). It is a five point assay consisting of two levels each of standard and unknown and one level HCG alone. The potency of an unknown is compared to that of a standard by determining the slopes of the dose response curves of each and obtaining a ratio from which the true potency can be ascertained.

METHOD OF CALCULATION

The following gives the detailed method of calculation together with a sample calculation of the potency of a preparation of FSH with reference to the standard 264-151-X.

Let $\sum w$ be the sum of the ovarian weights of p animals receiving 20 I.U. of HCG only (0 mg. of FSH).

$\sum x_1$ be the sum of the ovarian weights of m_1 animals receiving 20 I.U. of HCG and s_1 mg. of standard FSH.

$\sum x_2$ be the sum of the ovarian weights of m_2 animals receiving 20 I.U. of HCG and s_2 mg. of standard FSH.

$\sum y_1$ be the sum of the ovarian weights of n_1 animals receiving 20 I.U. of HCG and t_1 mg. of unknown FSH.

$\sum y_2$ be the sum of the ovarian weights of n_2 animals receiving 20 I.U. of HCG and t_2 mg. of unknown FSH.

As there are eight animals per level:

$$p = m_1 = m_2 = n_1 = n_2 = 8$$

Let N be the total number of animals:

$$N = p + m_1 + m_2 + n_1 + n_2 = 40$$

$\sum w = 346.5$ mg. $\quad \sum x_1 = 540.1$ mg. $\quad \sum x_2 = 895.4$ mg. $\quad \sum y_1 = 452.9$ mg. $\quad \sum y_2 = 567.4$ mg.

Let Z be the total of all ovarian weights:

$$Z = \sum w + \sum x_1 + \sum x_2 + \sum y_1 + \sum y_2$$
$$Z = 2802.3 \text{ mg.}$$

$$Ss^2 = \sum (s - \bar{s})^2 = m_1 s_1^2 + m_2 s_2^2 - \frac{(m_1 s_1 + m_2 s_2)^2}{N} = .08 + .32 - \frac{(.8 + 1.6)^2}{40} = .256$$

$$St^2 = \sum (t - \bar{t})^2 = n_1 t_1^2 + n_2 t_2^2 - \frac{(n_1 t_1 + n_2 t_2)^2}{N} = .08 + .32 - \frac{(.8 + 1.6)^2}{40} = .256$$

Only if $m_1 = n_1$, $m_2 = n_2$, $s_1 = t_1$, and $s_2 = t_2$ does $Ss^2 = St^2$.

$$Sst = \sum (s - \bar{s})(t - \bar{t}) = \frac{(m_1 s_1 + m_2 s_2)(n_1 t_1 + n_2 t_2)}{N} = \frac{(.8 + 1.6)(.8 + 1.6)}{40} = .144$$

$$Sxs = \sum (x - \bar{x})(s - \bar{s}) = s_1 \sum x_1 + s_2 \sum x_2 - \frac{(m_1 s_1 + m_2 s_2)(Z)}{N}$$

$$= (.1)(540.1) + (.2)(895.4) - \frac{(.8 + 1.6)(2802.3)}{40} = 64.95$$

$$Syt = \sum (y - \bar{y})(t - \bar{t}) = t_1 \sum y_1 + t_2 \sum y_2 - \frac{(n_1 t_1 + n_2 t_2)(Z)}{N}$$

$$= (.1)(452.9) + (.2)(567.4) - \frac{(.8 + 1.6)(2802.3)}{40} = -9.37$$

For convenience let

$$A = Ss^2 St^2 - (Sst)^2 = (.256)(.256) - (.144)^2 = .0448$$

The slope of the regression line of the standard preparation:

$$b_s = \frac{SxsSt^2 + SytSst}{A} = \frac{(64.95)(.256) + (-9.37)(.144)}{.0448}$$

$$b_s = 341.02$$

The slope of the regression line of the unknown preparation:

$$b_t = \frac{SxsSst + SytSs^2}{A} = \frac{(64.95)(.144) + (-9.37)(.256)}{.0448}$$

$$b_t = 155.22$$

The ratio of the slopes:

$$R = \frac{b_t}{b_s} = \frac{155.22}{341.02} = .4552$$

The potency of the unknown preparation is $R \times 100 = 45.52\%$ of the standard.

Let v be the within dose variance or mean square:

$$v = \frac{\text{Sum of squares of all ovarian weights} - \left[\frac{(\sum w)^2}{p} + \frac{(\sum x_1)^2}{m_1} + \frac{(\sum x_2)^2}{m_2} + \frac{(\sum y_1)^2}{n_1} + \frac{(\sum y_2)^2}{n_2}\right]}{(p - 1) + (m_1 - 1) + (m_2 - 1) + (n_1 - 1) + (n_2 - 1)}$$

$$v = \frac{235{,}653.63 - 217{,}571.57}{35} = 516.63$$

The standard error of R:

$$\sigma_R = \sqrt{\left[\frac{v}{b_s^2}\right]\left[\frac{Ss^2 - 2RSst + R^2 St^2}{A}\right]}^*$$

---

* Private communication with Dr. Finney confirmed the misprint "$+2RS_{x_s x_t}$" on page 101 of *Biological Standardization*.

$$\sigma_R = \sqrt{\left[\frac{516.63}{(341.02)^2}\right]\left[\frac{.256 - 2(.4552)(.144) + (.4552)^2(.256)}{.0448}\right]}$$

$$\sigma_R = .1328$$

Therefore the potency is $45.52 \pm 13.80\%$.

The formulas for the regression lines plotted in Figure 3 are:

$$Y_s = a + b_s s$$

$$Y_t = a + b_t t$$

$$\bar{s} = \frac{m_1 s_1 + m_2 s_2}{N} = \frac{.8 + 1.6}{40} = .06$$

$$\bar{t} = \frac{n_1 t_1 + n_2 t_2}{N} = \frac{.8 + 1.6}{40} = .06$$

$$a = \frac{Z}{N} - b_s\bar{s} - b_t\bar{t}$$

$$= \frac{2802.3}{40} - (341.02)(.06) - (155.22)(.06) = 40.28$$

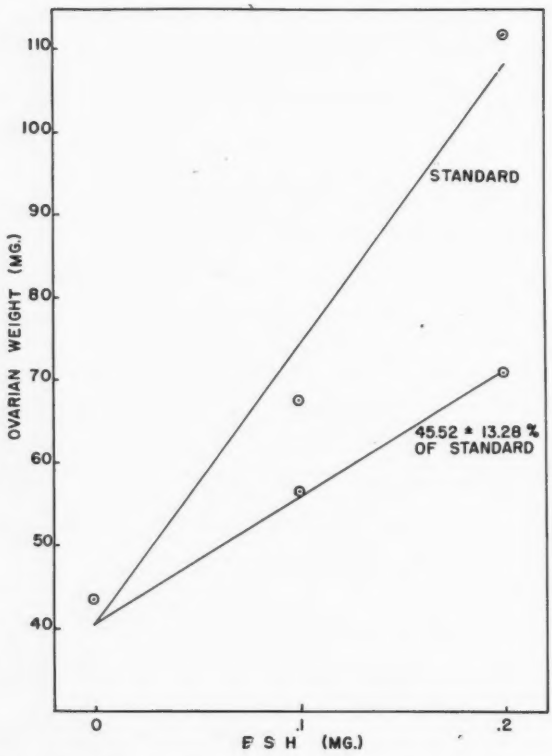

FIG. 3. Regression lines for the assay of a preparation of unknown potency of FSH against standard FSH.

$$Y_s = 40.28 + 341.02s$$
$$Y_t = 40.28 + 155.22t$$

For purposes of assay the 0.1 and 0.2 mg. doses of the standard were chosen as the levels at which the unknown should be matched. Each level should have the same number of animals to simplify the calculations. Using a two dose assay with eight animals per level potencies for unknown were determined with a standard error of 15 to 30%.

LIMITATIONS OF THE ASSAY

Due to the complexity of biological assays, and in particular assays of the pituitary hormones, it is essential to recognize the fact that there can be interfering substances. Of prime importance is knowing something about the sample to be assayed, i.e. degree of purity and contaminants. This is amply illustrated in the body weight and tibia assays for somatotropin (STH). It is almost impossible to accurately assay a sample which is low in STH activity and high in TSH or ACTH because these two substances will interfere with the response. In the case of the FSH assay reported above, it should be pointed out that the work was carried out on purified samples, and although the effects of the various hormones were tested, the effects of very high concentrations and of multiple contamination has not been ascertained. Indeed, the effects of high concentrations of hormones on all the pituitary hormone assays are not fully known.

If possible, a standard preparation should be run concomitantly with the unknown so that an adequate comparison may be made. This, no doubt, can be minimized by the selection of a highly inbred strain of rats.

In choosing a matching dose level for the unknown, the best results are obtained when the unknown response falls within the 0.1 to 0.2 mg. dose level range of the Armour Standard. The dose response curve has a fairly narrow range, 0.05 to 0.30 mg. of standard. At levels above 0.3 the dose response curve begins to plateau.

In summary, FSH may be simply and accurately assayed by the augmentation response to Human Chorionic Gonadotropin. The principal usefulness of this method should be in the assay of FSH preparations and not in the assay of minute amounts of FSH in other pituitary preparations.

SUMMARY

1. A simple assay for FSH using intact immature female rats and human chorionic gonadotropin has been developed. Two level assays gave potencies with a standard error of 15 to 30%.

2. Five anterior pituitary hormones when added in sizeable amounts to FSH did not interfere with the response.

Acknowledgments

The authors are indebted to Dr. Albert Segaloff of the Alton Ochsner Medical Foundation, New Orleans, Louisiana, and Dr. F. X. Gassner of Colorado A and M College, Fort Collins, Colorado, for their assistance and criticism. They also would like to express their appreciation to Curtis Johnson, William Frey and other members of The Armour Laboratories Biological Control Section for their assistance in carrying out the numerous animal experiments.

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ERRATUM

In the paper "Factors Affecting the *in vitro* Metabolism of Adrenal Steroids by Liver Tissue" by H. Bacchus which appeared in ENDOCRINOLOGY 53:617, 1953, the ordinate of Figure 2 should be read in units of 600 instead of 1000 K_{sp}.

FACTORS AFFECTING THE *IN VITRO* METABOLISM OF ADRENAL STEROIDS BY LIVER TISSUE

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IT HAS been demonstrated that the rise of urinary 17-ketosteroids following injection of cortisone acetate into adrenalectomized female rats is prevented by simultaneous ascorbic acid treatment (Bacchus, Altszuler, and Heiffer, 1952). Subsequent work indicated that ascorbic acid treatment is also capable of increasing the urinary corticosteroids following cortisone injection into such animals, while depressing the 17-KS excretion (Bacchus and Heiffer, 1953). In a similar manner it was observed that the hematologic effects of injected cortisone are prolonged by simultaneous ascorbic acid treatment (Bacchus, Altszuler and Heiffer, 1952a). These data were interpreted as suggesting that ascorbic acid depresses the breakdown or inactivation of cortisone. This suggestion has recently received support from our observation that the liver tissues of ascorbic acid-treated rats metabolize cortisone to a lesser extent than do control liver tissues (Bacchus, 1953).

This paper reports further data on the metabolism of cortical hormones by *in vitro* preparations of rat liver and on the relationship of ascorbic acid to this process.

MATERIALS AND METHODS

In the experiments known amounts of adrenal steroids were incubated with liver tissue. The content of the reactive structures in these preparations after three hours of incubation was determined by various methods. These final contents were compared to the amounts originally introduced, and the amount of loss was determined.

The preparations, incubation and extraction procedures were similar to those used previously (Bacchus, 1953).

Tissue preparations: Male rats (200–300 gm.) were anesthetized with nembutal, laparatomy was performed and the liver excised and washed twice in phosphate saline buffer. Sixty milligram slices were weighed and placed in the incubation vessels containing steroid and buffer.

Steroid preparations: Cortisone acetate and cortisone were used in Experiments 1, 4 and 5. Measured volumes of the suspensions were dispersed in phosphosaline buffer. The total volume placed in each tube was three ml. containing 200 micrograms of cortisone or of cortisone acetate. In Experiments 2, 3 and 5 desoxycorticosterone acetate

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and desoxycorticosterone were used. Weighed amounts of steroid were dissolved in small amounts of absolute ethanol. Aliquots of this were distributed in warmed phosphosaline buffer. Each three ml. contained 200 micrograms of steroid.

Buffer: Phosphate buffer (pH 7.4) was made and checked by pH meter. A 1:1 mixture of buffer and saline was used.

Incubation: Except where indicated, the incubations were conducted for three hours at 38° C.

Extraction: The tissue and incubate were extracted with chloroform. Seven ml. of chloroform were added to each incubation vessel. These were then shaken by hand 100 times. The tubes were then centrifuged for five minutes at the end of which the supernatant aqueous buffer layer was aspirated off. The thin layer of tissue protein appeared at the interface. Two ml. of distilled water were added to each tube, which was then shaken and centrifuged. The aqueous layer was aspirated off. Small amounts of anhydrous sodium sulfate were added to each tube. The tubes were again centrifuged. The chloroform extract was then quantitatively poured into separate tubes. The incubation tubes were rinsed with chloroform. The chloroform extract was evaporated to dryness in a vacuum desiccator at 38° C. The extracts were dissolved in methanol and aliquots were used for analytic procedures.

Analytic procedures:

a) Ultraviolet absorption at 240 mμ.

Samples of the extracts were diluted with methanol. The optical densities were studied at 240 mμ in a Beckman Model DU Spectrophotometer. The optical densities (at 240 mμ) of the unknown samples were compared with those of standard steroid samples and the amounts of steroids in the extracts determined. This method has been used by other workers (Schneider and Horstmann, 1951, 1952). Data on the validity of this procedure will be provided in the "Control Experiments" section. The optical density reading at 240 mμ is regarded as indicative of the conjugated unsaturated system (Δ4,3-ketone) of the steroid nucleus (Haines, 1952; Schneider and Horstmann, 1951, 1952).

b) Porter-Silber Assay—for 17-OH, 20, 21-ketol group of the side chain. One ml. aliquots of the methanol extracts were employed for the procedure described by Porter and Silber (1950). The complete series of chemical controls recommended by these workers was employed in these experiments.

c) *Heard-Sobel Procedure for Phosphomolybdic Acid Reducing Substances.* Aliquots of methanol extracts were evaporated to dryness in a vacuum desiccator at 38° C. The Heard and Sobel (1946) procedure for P.R.L. substances was conducted on these extracts. This method is regarded as indicative of the Δ4,3-ketone group and of the αketol side chain.

d) *The Sulfuric Acid Chromogen Procedure of Zaffaroni* (1950) was employed in Experiment 4. Methanol extracts were evaporated to dryness. Three ml. concentrated sulfuric acid were added to the extracts. The tubes were stoppered and left at room temperature for two hours. The optical densities of these preparations were studied in the Beckman Model DU Spectrophotometer over the range 250–500 mμ. The significance of this procedure will be discussed later.

CONTROL EXPERIMENTS

Extraction of the liver slice-hormone preparation before incubation showed high recovery values. Porter-Silber assay revealed a 90–95% recovery of cortisone and of cortisone acetate. In studies with DOC and DCA

extraction and Heard-Sobel analysis of such preparations revealed a similar range of recoveries. Liver tissue incubated without steroids was also extracted and analyzed. Such preparations showed no endogenous steroids. Chloroform extracts of preparations containing ascorbate did not exhibit significant optical density readings by these methods. The Porter-Silber and the Heard-Sobel procedures have been used in previous experiments (Bacchus, 1953; Bacchus and Heiffer, 1953a, 1953b). Reproducibility and recovery studies demonstrated these methods to be highly reliable.

Ultraviolet spectroscopy: Extraction of the steroid-liver preparations before incubation showed a 90–106% recovery by this method. Liver tissue incubated without the hormones showed negligible optical densities over the range of 220–260 mμ. Liver blanks were nevertheless routinely used in these experiments. Extracts of ascorbate preparations with and without liver tissue showed significant optical density readings (Fig. 1). Washing with water completely eliminated this (Fig. 1). Unwashed extracts failed to exhibit significant optical density readings with the Porter-Silber and

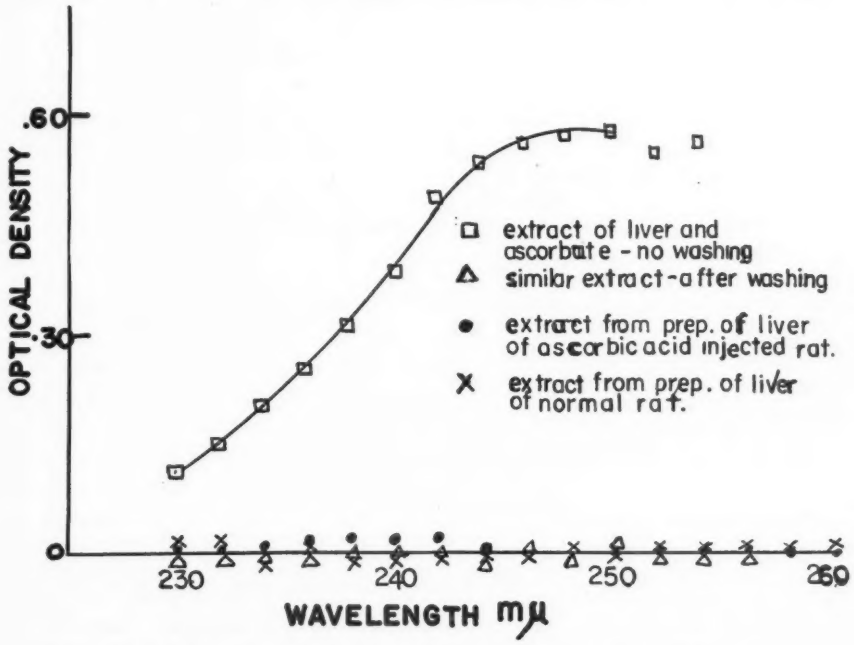

FIG. 1. Optical densities of extracts of incubation preparations. Preparations were extracted with chloroform, washed (except where indicated in figure), dehydrated, and evaporated *in vacuo*. Methanol was used as the solvent for the extracts. Methanol blank was employed in these studies.

the Heard-Sobel procedures. The washing procedure was nevertheless adopted in all experiments.

The ultraviolet absorption of the various steroids used were studied over the range 220–260 mμ on the Beckman spectrophotometer. The optical densities of representative extracts were also studied. The maximum absorption of the pure steroids and of the steroid-containing extracts occurred at 240 mμ. The optical density readings of the extracts at 240 mμ were, therefore, compared to those of the appropriate standard steroids and the amounts of steroids determined.

The specific extinction coefficients (K_{sp}) of the standard steroid samples were calculated on the basis of the observed optical densities, and the *known* concentrations. The K_{sp} of the extracts were based on the observed optical density readings, and the *calculated* concentrations.[1] Figure 2 shows typical absorption curves. It is seen that the absorption curves for the extracts are identical, within experimental error, to that of the standard cortisone preparation. Similar observations were made with cortisone acetate, desoxycorticosterone, and desoxycorticosterone acetate and the respective extracts.

These observations suggest that liver tissue, and ascorbate, do not exert significant interference to the analytical methods used. The data also render the employment of the maximum optical density at 240 mμ as a measure of the reactive structure of the steroid. Further qualitative data are presented in Experiment 4.

EXPERIMENTS AND RESULTS

I. *Metabolism of cortisone acetate*

Liver tissue (60 mg.) was obtained from adult male rats (200–300 gm.) under the following conditions:

Group 1 rats were fed *ad libitum* Purina Laboratory Chow and tap water. Over the 24-hour period immediately preceding procurement of the liver samples, these rats received small amounts of isotonic saline by two intraperitoneal injections spaced 12 hours apart. Group 2 rats were on the

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[1] The calculation of the K_{sp} (O.D. reading/gm./ml. × thickness (cm)) of the extracts is considered to be valid for the following reasons. (a) the concentrations of the steroid in the extracts were calculated from the optical density readings at 240 mμ compared to that of standard steroid at the same wavelength. This was the observed absorption maximum in both cases. This procedure is considered valid in view of the occurrence of these maxima. (b) The agreement between the calculated K_{sp} of the standard steroids and that of the extracts is further indication of the validity of the procedure. The procedure is employed in this paper merely as an indication of the identity of the steroid in each extract.

same fare as those of Group 1, but received a total of 200 mg. sodium ascorbate in two intraperitoneal injections during the preceding 24-hour period. Liver tissue was excised while the rats were under nembutal anesthesia and eight hours after the last vitamin injection. The following incubation preparations were employed:

Set (a): Liver of Group 1 rats incubated with 200 micrograms cortisone acetate; Set (b): Liver of Group 2 rats incubated with hormone; Set (c): Liver of Group 1 rats with hormone plus 0.03*M* sodium ascorbate added *in vitro*; Set (d): Hormone plus liver (normal or Group 2), some were extracted before, and others after, incubation; Set (e): Hormone without liver tissue; Set (f): Ascorbate in buffer; and Set (g): Liver alone in buffer.

After three hours of incubation the preparations were extracted, washed and evaporated. Methanol solutions were used for Porter-Silber Assay and for the ultraviolet absorption studies. Standard hormone solutions were made up of cortisone acetate. The data are presented in Table 1. The Porter-Silber Assay data indicate a 50% loss of cortisone acetate occurs when the hormone is incubated for three hours with liver tissue of normal

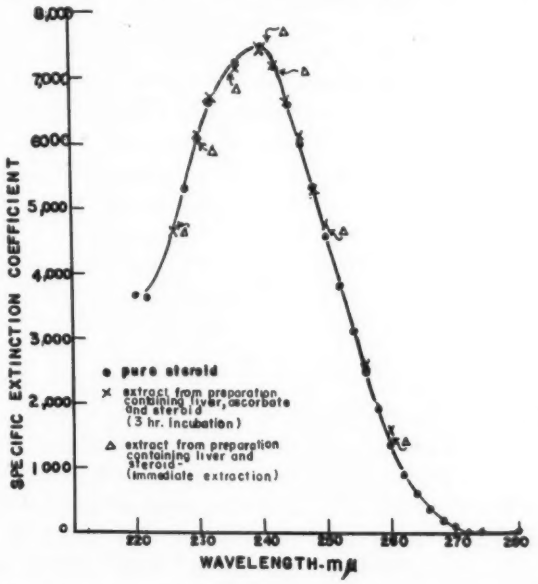

FIG. 2. Ultraviolet absorption spectrum of cortisone and of extracts of liver-plus-cortisone preparations. Specific extinction coefficients calculated as described in text. Similar patterns were observed with DOC, DCA, and cortisone acetate and their respective steroid-liver extracts. Spectrum studied on a Beckman Model DU Spectrophotometer. Spectrum of extract of liver is shown in Fig. 1.

TABLE 1. METABOLISM OF CORTISONE ACETATE BY *in vitro* PREPARATIONS OF LIVER SLICES FROM ASCORBATE-TREATED, AND CONTROL, MALE RATS

Treatment	Micrograms loss/60 mg. liver/3 hr.	
	17-OH, 20,21-ketol Porter-Silber assay	Δ^4, 3-ketone Ultraviolet absorption
1. Control (saline inj.)	101 ± 4.3 (15)	126 ± 6.7 (6)
2. Ascorbate-injected rat	71* ± 3.2 (15)	78* ± 11 (6)
3. Control rats' liver tissues plus ascorbate (0.03 *M*)	102 ± 4.9 (15)	81* ± 8 (6)
4. Liver and cortisone acetate (extracted immediately)	Recovery 182–204 μgm.	Recovery 190–212 μgm.
5. Boiled liver and cortisone acetate (extracted after incubation)	Recovery 180–196 μgm.	186 μgm.
6. Liver tissue only	0	7 μgm. (rec.)
7. Ascorbate (0.03 *M*)	0	0

60 mg. liver tissue incubated with 200 micrograms cortisone acetate in phosphate-saline buffer (pH 7.40), for 3 hr. at 38° C. System extracted with chloroform.

* Significantly different from control ($P < 0.05$ or $P < 0.01$).

Standard Errors $\left(\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}\right)$ follow the mean values.

Figures in parentheses indicate numbers of animals used.

rats. The loss of steroid is significantly less ($P = < 0.01$) when liver tissue from ascorbic acid-injected rats is used. *In vitro* addition of ascorbate to normal liver preparations does not alter the extent of loss (50%).

Ultraviolet studies for the Δ4,3-keto component of the steroid indicate that this structure is metabolized to the extent of 63% over the three hour period. The loss of this structure is significantly less when liver tissue from ascorbic acid-treated rats is used ($P = < 0.01$). In contrast to the failure of the *in vitro* ascorbate to depress the loss of the Porter-Silber positive group (side chain), the loss of the Δ4,3-ketone group is significantly depressed by the *in vitro* addition of ascorbate to liver and hormone preparations. The control determinations indicated that the liver blanks contain only minimal amounts of reactive substances; fairly high recovery values were obtained from recovery studies.

The data of this experiment reveal that the injection of ascorbic acid to normal rats confers an ability to the liver tissue to depress the metabolism of cortisone acetate, in comparison to that of control liver. This depression affects both the Δ4,3-ketone, and the 17-OH, 20, 21-ketol groups. *In vitro* addition of ascorbate to normal liver depresses the rate of loss of the Δ4,3-ketone group, but not of the side chain.

II. *Metabolism of Desoxycorticosterone acetate*

A similar experimental set-up as in Experiment 1 was employed. The steroid substance used here was desoxycorticosterone acetate. The analyti-

TABLE 2. METABOLISM OF DESOXYCORTICOSTERONE ACETATE BY *in vitro* PREPARATIONS OF LIVER SLICES FROM ASCORBATE-TREATED, AND CONTROL, MALE RATS

Treatment	Micrograms loss/60 mg. liver/3 hr.	
	Phosphomolybdic acid Reducing substances (PRL)	Δ^4,3-ketone U-V at 240 mμ
Control (saline inj.)	115 ± 4.1 (n = 10)	122 ± 4.4 (n = 11)
Ascorbate-injected	93 ± 4.9* (11)	74 ± 9* (7)
Control rats' liver tissues plus ascorbate (0.03*M*)	82 ± 12.2* (12)	98 ± 4.4* (6)
	Recoveries-micrograms†	
Liver and DCA (extracted immediately)	188 μgm.	202
Boiled liver and DCA (incubated 3 hr.)	180	190
Liver tissue only	3	0
Ascorbate (0.03 *M*)	0	0

60 mg. liver tissue incubated with 200 micrograms DCA in phosphate-saline buffer (pH 7.40) for 3 hr. at 38° C. System extracted with chloroform.

* Significantly different from control ($P < 0.05$ or $P < 0.01$).

Figures in parentheses indicate numbers of animals used.

† Porter-Silber Assay revealed no 17-OH,20,21-ketol substances in these preparations.

cal procedures employed were the Porter-Silber Assay, the Heard-Sobel procedure and ultraviolet spectroscopy. Desoxycorticosterone acetate was used as the standard in the analytical procedures. The data are presented in Table 2. No 17-OH, 20, 21-ketol substances were observed in any of these preparations. The Heard-Sobel method revealed that significantly smaller amounts of the steroid are metabolized when liver tissue of ascorbic acid injected rats is used. Similarly the depressed metabolism is observed when ascorbate is added to *in vitro* preparations of normal liver tissue. A similar relationship is shown by the ultraviolet studies. Good recovery values were observed. Blank and control preparations revealed negligible interference of the analyses by liver and by ascorbate.

III. *Metabolism of desoxycorticosterone (free alcohol)*

Liver tissue from four normal rats was used in this study. One set of incubations contained normal tissue and added hormone in buffer, another series containing hormone, buffer and added ascorbate (0.03*M*). Control vessels contained liver tissue and hormone (extracted before incubation), liver tissue and ascorbate, incubated and non-incubated. Aliquots of these extracts were used for the Heard-Sobel procedure and for ultraviolet studies, in both cases desoxycorticosterone (alcohol) was used as the standard. The data are presented in Table 3. The data indicate that the metabolism of the structures studied is depressed by the *in vitro* addition of 0.03*M* ascorbate.

The data from Experiments 2 and 3 do not provide information on the

TABLE 3. METABOLISM OF DESOXYCORTICOSTERONE BY *in vitro* PREPARATIONS OF LIVER SLICES

Treatment	Micrograms loss/60 mg. liver/3 hr.	
	Phosphomolybdic acid Reducing substances	Δ^4,3-ketone
Normal	106 ± 8.8 (n = 4)	133 ± 5.7 (4)
Normal liver plus 0.03 *M* ascorbate added	46 ± 10* (4)	67 ± 4* (4)
	Recoveries-micrograms	
Desoxycorticosterone and liver tissue (immediate extraction)	186	206
Liver tissue only	5	0

* Significantly different from control.

metabolism of the ketol side chain. The Heard-Sobel procedure measures both the αketol group and the Δ4,3-ketone group. In view of this it is difficult to study the selectivity of the action of ascorbate, and the method of administration, as is possible in the studies involving the Porter-Silber positive group, and the Δ4,3-ketone in cortisone.

IV. *Metabolism of cortisone (free alcohol)*

Porter-Silber assay and ultraviolet absorption studies were conducted on extracts of preparations as in Experiment 1. Vessels were extracted at zero hour, one hour, two hour and three hour of incubations. The data are presented in Figures 3 and 4. Appropriate controls were run.

The Sulfuric Acid Chromogen- Procedure- of Zaffaroni (1950): Aliquots of methanol extracts were evaporated in vacuo at 38° C. Sulfuric acid was added to these extracts and the Zaffaroni sulfuric acid chromogen procedure conducted. The absorption of these solutions from 250–500 mμ was studied. Cortisone has been reported to exhibit maxima at 280, 343 and 410 mμ. (Zaffaroni, 1950). Our observations confirmed these maxima. (Fig. 5). In addition all of the extracts studied also exhibited the maximum at 280–285 mμ and at 410 mμ. In the case of the extract of the preparation containing liver, cortisone and ascorbate, the peak at 340–345 mμ was attenuated. In all of the other preparations with steroid this peak was still intact. It has been suggested that the peak at 280 mμ is clearly due to the conjugated unsaturated system. (Schneider and Horstmann, 1952). Quantitative comparisons made at this maximum revealed a 37% loss of the structure when the normal liver is incubated with cortisone. Ascorbate addition is shown to minimize this loss (Fig. 5). Further quantitative studies with this method were not done. This experiment provides additional qualitative data on the identity of the steroid in extracts of the incubation preparations.

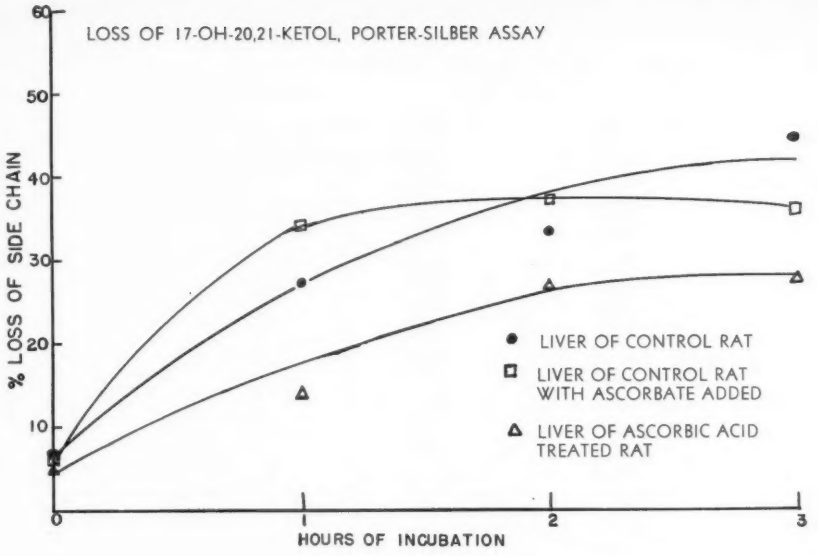

FIG. 3

FIGS. 3 and 4. Cortisone was incubated with liver tissue of ascorbate-treated rats, with liver of control rats, with and without added ascorbate. Extractions were done at 0, 1, 2, and 3 hours of incubation.

$$\text{Per cent loss} = \left(\frac{\mu\text{gm. cortisone in tube initially} - \mu\text{gm. recovered}}{\mu\text{gm cortisone in tube initially}} \times 100\right)$$

Side chain (17-OH, 20, 21-ketol) measured by Porter-Silber method. Δ^4,3-ketone measured by ultraviolet absorption at 240 mμ. Three animals in each group.

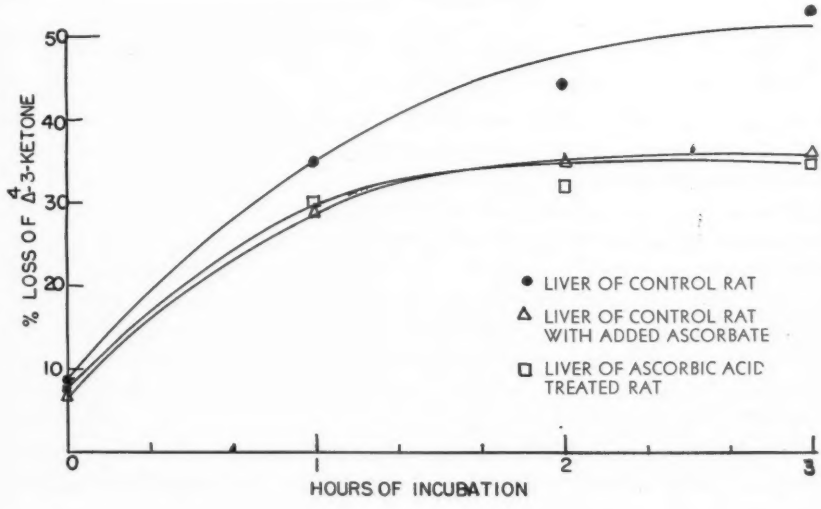

FIG. 4

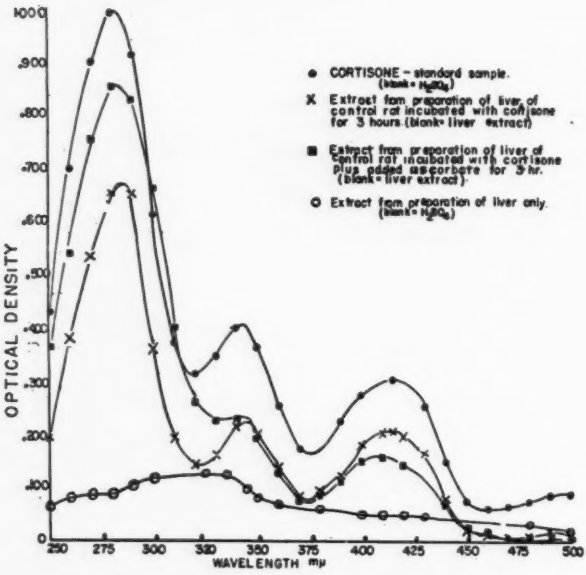
OPTICAL DENSITY
WAVELENGTH mμ
CORTISONE - standard sample.
Extract from preparation of liver of control rat incubated with cortisone for 3 hours (blank = liver extract)
Extract from preparation of liver of control rat incubated with cortisone plus added ascorbate for 3 hr. (blank = liver extract).
Extract from preparation of liver only.

FIG. 5. Sulfuric Acid-Chromogen Procedure of Zaffaroni. The absorption spectra of various representative extracts following the addition of concentrated sulfuric acid. Optical density readings were made between 250–500 mμ on the Beckman Model DU Spectrophotometer at 2 mμ intervals. Extracts of preparations containing no liver tissue were compared to the sulfuric acid blank. Extracts of liver-containing preparations were compared to the liver-extract blank. The liver-extract is seen to exhibit some observable density readings when compared to the H_2SO_4 blank.

V. *Effect of Nitrogen atmosphere and of KCN (0.01M) on the metabolism of cortisone acetate and of desoxycorticosterone*

Incubation preparations containing normal liver and steroid in buffer were flooded with nitrogen and stoppered. KCN (0.01*M*) was added to other preparations. Tissue blanks as described previously were also employed in this study. The data are presented in Figure 6. It is observed that the metabolism of the 17-OH, 20, 21-ketol side chain is not appreciably altered by either N_2 or KCN. Both these factors markedly and significantly depress the metabolism of the Δ4,3-ketone. Schneider and Horstmann (1951) also observed that N_2 depressed the metabolism of the conjugated unsaturated system, but not of the side chain.

VI. *Supplementary study*

In a supplementary study extracts of (a) cortisone and liver, (b) cortisone, liver, and ascorbate, (d) liver-and-ascorbate, were applied to impreg-

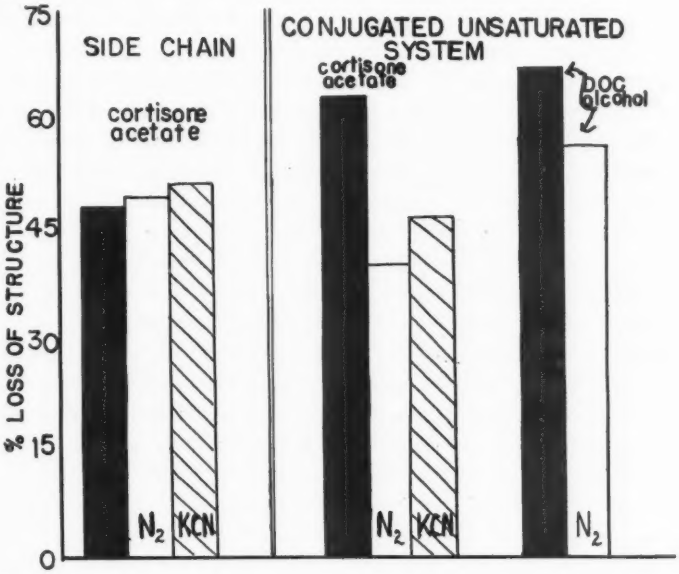

FIG. 6. Effect of nitrogen atmosphere, and of KCN on the metabolism of the steroid side chain and of the conjugated unsaturated system. Solid columns refer to preparations in air phase. Open columns (N_2) refer to preparations flooded with nitrogen. Shaded columns refer to preparations to which KCN was added. Four determinations per group. Per cent loss of structure refers to the loss of the side chain group measured by Porter-Silber assay, or of the conjugated unsaturated system measured by ultraviolet absorption at 240 mμ.

nated paper strips and chromatographed in the propylene-glycol-toluene system (Burton *et al.*, 1950). Control strips were treated with cortisone and desoxycorticosterone. The steroid positions were identified by the iodine reagent, and by scanning in ultraviolet light (Haines, 1953). In all of the steroid-containing extracts the positions corresponded to that of cortisone. Eluates of these positions from duplicate strips revealed optical densities at 240 mμ. Quantitative studies were not conducted. These data provide additional evidence on the steroid examined. Liver tissue did not show any steroids.

DISCUSSION

In the experiments described it was necessary to employ precautions to minimize the possible contribution of tissue extractives and hormone metabolites. The precautions taken were described. Remarkable reproducibility of the findings were observed. For example, it is seen that the metabolism of Δ4,3-ketone group proceeds to the extent of 120–133 microgm.

/three hours in all of the preparations. Also, there is agreement in the rate of loss of the side chain group. The data indicate that ascorbate injection into intact rats is capable of depressing the *in vitro* metabolism of cortical hormones by liver slices. Data regarding the effect of ascorbic acid nutrition on the metabolism of the 17-OH, 20, 21-ketol side chain (Porter-Silber Assay) have been obtained previously (Bacchus, 1953). Those data are in agreement with the observations in the present study.

It is evident from these data that the extent of metabolism of the Δ4,3-ketone group, and of the side chain, over the three hour period, is different. Differences in the rates of loss of these groups have been shown by Schneider and Horstmann (1951, 1952). The present data confirm those observations. Furthermore, it is seen that the various factors employed exert selective effects on the groups. Ascorbate, nitrogen atmosphere, KCN, and injection of ascorbate all depress the metabolism of the conjugated unsaturated system. The metabolism of the 17-OH, 20, 21-ketol side chain, on the other hand, is depressed only by the injection of ascorbate into the animal. It was suggested by Schneider and Horstmann (1951) that data on selective influences may aid in elucidating the mechanism of the loss of these groups.

The data suggest that some vital process, involving *in vivo* transformations, is involved in the depression of the metabolism of the 17-OH, 20, 21-ketol side chain. Ascorbate added *in vitro* fails to depress the metabolism of the side chain. The metabolism of the conjugated unsaturated system is observed to be depressed in nitrogen atmosphere. This observation suggests that an oxidative process is involved in this mechanism. It has been shown (Schneider, 1952) that the conjugated unsaturated system is probably inactivated by reduction. It is possible then, that some intermediary substance is oxidized to permit this reduction. Nitrogen atmosphere, in depressing the oxidative process, therefore, prevents the reduction of the Δ4,3-ketone.

If the suggestion (Schneider and Horstmann, 1951) that different enzymes are involved in the metabolism of the two groups is correct, then certain qualitative conclusions regarding the enzyme systems are permissible from the present study. It is likely, then, that the enzyme system responsible for the reduction of the conjugated unsaturated system is inhibited by ascorbate, *in vivo* or *in vitro*, by KCN, and by N_2. Similarly, it may be said that the enzyme system responsible for the metabolism of the side chain is inhibited only when ascorbate is injected into the live animal.

These *in vitro* data may be only suggestive of *in vivo* processes. The data presented here receive support from *in vivo* observations suggesting that ascorbic acid suppresses the excretion of 17-ketosteroid metabolites of

injected cortisone. Other *in vivo* observations suggest that the urinary corticosteroid (PRL) excretion is greater in rats receiving ascorbate along with cortisone. This was associated with a prolongation of the eosinopenic and lymphopenic actions of the injected hormone.

Experiments presented here suggest that the urinary steroid excretion may be indicative of liver function, as well as of the functions of the endocrine organs.

SUMMARY AND CONCLUSIONS

The metabolism of adrenal steroids by liver slices in *in vitro* preparations was studied. The Porter-Silber Assay was employed to detect the 17-OH 20, 21-ketol group, the Heard-Sobel procedure for the αketol and/or the Δ4,3-ketone, and ultraviolet absorption at 240 mμ for the Δ4,3-ketone group. Control experiments are described in which it is shown that liver tissue, or ascorbate fail to interfere significantly with the methods. The data indicate that ascorbate injection into rats confers an ability to the liver to depress the metabolism of adrenal steroids. Ascorbate added *in vitro* to liver steroid preparations depresses the metabolism of the conjugated unsaturated system, but not of the 17-OH, 20, 21-ketol group. The latter group is affected only by injected ascorbate. N_2 and KCN depress the metabolism of the conjugated unsaturated group.

The data are discussed in relation to *in vivo* studies which suggested that ascorbic acid may depress the metabolism of adrenal steroids.

Acknowledgement

The author gratefully acknowledges the generosity of Ciba Pharmaceutical, Inc. for supplying the DOC and DCA and of Merck and Co. for supplying cortisone and cortisone acetate. Doctor P. K. Smith, Professor of Pharmacology, kindly made available certain facilities for some of the chemical work. Part of this work was supported by Grant H-1170 from the National Heart Institute.

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THE EFFECT OF ANESTHETIC AGENTS ON THE THYROID ACTIVITY OF THE RAT[1]

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INTRODUCTION

DURING a course of studies involving the measurement of thyroid activity by radio iodide uptake in anesthetized animals, observed differences appeared to be related to the kind of anesthetic agent employed. Reduced I^{131} uptake was frequently noted especially when thiopental sodium (pentothal) was used. Previously, Williams *et al.* (1949) have reported erratic results in the measurement of radio iodide uptakes by rat thyroid when thiopental sodium was administered. This may have been due to an anti-thyroid effect of the drug. The anti-thyroid activity of the thiobarbituric acids has been demonstrated by McGinty and Bywater (1945). The thiobarbituric acids tested by these investigators were less active than thiouracil except 5,5-diethyl-2-thiobarbituric acid which had an increased anti-thyroid property. Astwood (1943) in a 10 day feeding experiment, has reported inhibition of thyroid function by thiobarbituric acids. Bartels (1945) has used thiobarbiturates in the treatment of hyperthyroidism.

The studies reported here are the results of our preliminary investigations of the effects of anesthetic agents on thyroid function.

EXPERIMENTAL

Adult male rats of the Wistar strain, weighing from 150 to 210 gms. were used in groups containing no less than eight animals. Prior to use, the rats were fed commercial mixed grain rations and water, ad libitum. At zero time, the various anesthetic agents were administered intraperitoneally at the dose levels indicated in the table below. The levels used were those found to be most useful for producing short term anesthesia. Ten to fifteen minutes after injection, when the anesthesia became effective, carrier-free radio iodide, dissolved in buffered isotonic saline, was administered

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intraperitoneally. The activity injected in these experiments ranged from 5 to 8 μc I^{131} per kgm. body weight. Four hours later, the rats were sacrificed by exsanguination, the entire thyroid glands removed, stripped free of fascia and adherent tissue, weighed to the nearest 0.2 mgm. and plated for radiological assay. Radio assay was performed with the aid of an end-window Geiger detector (1.4 mgm./cm.^{2}) and appropriate scaler. The overall efficiency of the system was 4.3% Counting was continued to give a statistical error of two per cent or less. Results are expressed as counts per minute (c.p.m.) per milligram of thyroid tissue.

Rats not given anesthetic agents but only I^{131} served as controls for each experiment. Separate groups of controls were run each time an agent or group of agents were tested.

RESULTS

The data presented in the table indicate that nembutal,[2] the sedative drug most often used in these laboratories, had no pronounced effect upon the iodide concentrating property of the normal rat thyroid. Delvinal[2] and avertin,[2] likewise did not interfere significantly with this mechanism. Thiopental,[2] however, reduced the efficiency of the iodine binding mechanism by 82 to 89%.

DISCUSSION

The minor effects produced by nembutal, delvinal and avertin are probably non-specific and due essentially to slightly impaired circulatory and respiratory processes that follow sedative action. Nembutal and delvinal have a similar molecular geometry, whereas, avertin is quite different structurally. Since all three agents produce insignificant effects on I^{131} uptake, their action cannot be related to structure. Thiopental has a molecular geometry similar to nembutal and delvinal but it can be considered to be a substituted thiourea. The thioureas are well known for their anti-thyroid action (Pitt-Rivers, 1950) and the thiopental effect demonstrated here was anticipated. Studies on the metabolism of thiopental by Taylor (1950, 1952), Bollman (1950) and Brodie (1950, 1952) indicate it to be diversely attacked by the organism, thiourea being one of the products of its metabolism. It may be that the thiourea so produced is the ultimate effective anti-thyroid agent. This possibility is being tested currently by *in vitro* studies of the iodide fixing property of thyroid tissue in the presence of thiopental and products obtained from incubating thiopental with various tissue homogenates. The data obtained in this experiment

[2] Nembutal is sodium 5-ethyl-5-(1-methylbutyl) barbiturate. Delvinal is sodium 5-ethyl-5-(1-methyl, 1-butenyl) barbiturate. Avertin is tribromoethanol. Thiopental sodium (Pentothal, Abbott) is sodium 5-ethyl-5-(1-methylbutyl) thiobarbiturate.

THE EFFECT OF ANESTHESIA ON THE I^{131} FIXING MECHANISM OF THE RAT THYROID

Expt. No.	Drug	Dose	Average cpm./mgm. thyroid	Per cent of control†
1	None		157 ± 14*	100.0
	Nembutal	25 mgm./kg.	144 ± 9	91.7
	Nembutal	50 mgm./kg.	143 ± 17	91.0
2	None		119 ± 20	100.0
	Thiopental	40 mgm./kg.	19 ± 3	16.0
	Thiopental	70 mgm./kg.	13 ± 3	10.9
3	None		203 ± 19	100.0
	Thiopental	40 mgm./kg.	42 ± 10	20.7
4	None		256 ± 17	100.0
	Nembutal	25 mgm./kg.	243 ± 21	94.9
	Delvinal	40 mgm./kg.	259 ± 43	101.2
	Thiopental	40 mgm./kg.	47 ± 11	18.4
	Avertin	60 mgm./kg.	237 ± 25	92.6

* Standard error of the mean.
† Based on mean values.

suggest that thiobarbiturates be avoided in studies involving thyroid function, especially diagnosis via radio-iodide technique.

SUMMARY

Thiopental markedly reduces I^{131} uptake by thyroid tissue *in vivo*. Nembutal, delvinal and avertin exercise no significant effects on the thyroid system studied. It is probable that metabolic products of thiopental are responsible for the observed anti-thyroid action.

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FURTHER OBSERVATIONS ON THE HYPERTENSIVE PROPERTIES OF COMPOUND F ACETATE IN THE RAT[1]

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RECENTLY, in screening various compounds for their potential ability to inhibit the cardiovascular-renal effects of desoxycorticosterone acetate (DCA) we observed that Compound F acetate effected a considerable elevation in blood pressure (Friedman *et al.*, 1952). Indeed, matched against DCA in young rats, it seemed a more potent hypertensive agent than the synthetic steroid. Interestingly, unlike DCA, Compound F did not appear to cause any well-defined electrolyte shift. Masson *et al.* (1952) have also noted the hypertensive property of Compound F.

Because of the possible implications of this natural steroid of the adrenal in theories concerning the rôle of the adrenal cortex in clinical hypertensive disease, it seemed of importance to determine further points of similarity and contrast between DCA and Compound F. In particular, the present report is concerned with the effect of the various sensitizing procedures commonly used to intensify DCA hypertension, with the possible rôle of the kidney, and with the effect of Compound F in the adrenalectomized animal.

METHODS

Male albino rats of an inbred Wistar strain were used throughout. Compound F acetate (Hydrocortone acetate-Merck) was administered by subcutaneous injection as a saline suspension of microcrystals, 25 mg./cc., with added benzyl alcohol as preservative. Blood pressures were determined indirectly by tail plethysmography and directly using a Sanborn electromanometer coupled with a 22 gauge needle to the aorta, or where indicated, with a 24 gauge needle to the femoral artery. All blood pressure determinations were carried out on animals lightly anesthetized with ether. Plasma electrolytes were determined using a Beckman model B flame photometer. At the conclusion of each experiment the animals were sacrificed and organs weighed after 24 hours fixation. Histological sections were studied after trichrome staining.

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[1] With the aid of a grant from the National Research Council of Canada and the Schering Corporation.

Experiment 1. The effect of saline and of uninephrectomy on Compound F hypertension

In previous experiments using young rats given 2 mg. of Compound F acetate daily it had been noted that marked interference with body growth occurred (Friedman *et al.*, 1952). For the present experiment, where potential intensifying procedures were to be used, it was thought advisable to reduce the dose of the steroid and to use larger rats. Accordingly, 48 rats of an initial average weight of 175 grams were selected for division into 6 groups of 8 rats each. Group 1 served as untreated intact controls, group 2 received 1 mg. of Compound F acetate per day and group 3 received a like amount of this steroid together with the substitution of 1% saline as drinking water. Groups 4, 5 and 6 were all subjected to unilateral nephrectomy one week prior to the experiment. Group 4 received no further treatment, group 5 received 1% saline in place of drinking water and group 6 received both Compound F acetate as above and saline in place of drinking water. Table 1 summarises the pertinent data.

TABLE 1

Group	Intact control	Compound F	Compound F + saline	Uninephrectomy control	Uninephrectomy + saline	Compound F + uninephrectomy +saline
Initial body wt., gms.	185 ± 4.2	183 ± 3.6	183 ± 5.0	163 ± 6.3	172 ± 5.5	166 ± 6.3
Final body wt., gms.	281 ± 14.8	245 ± 21.6	202 ± 9.8	267 ± 7.9	267 ± 7.0	218 ± 13.7
Plasma Na mEq	148 ± 5.7	154 ± 6.6	161 ± 5.7	156 ± 3.7	155 ± 3.1	146 ± 1.7
Plasma K mEq	4.0 ± 0.2	4.3 ± 0.9	4.9 ± 0.1	4.4 ± 0.1	4.8 ± 0.2	5.2 ± 0.1
Direct Aortic Blood Pressure						
Systolic	109 ± 3.1	125 ± 3.5	121 ± 1.6	118 ± 14.0	104 ± 4.2	124 ± 6.0
Diastolic	79 ± 5.4	89 ± 6.2	82 ± 4.3	78 ± 2.8	70 ± 5.0	87 ± 5.5
Mean	90 ± 4.2	102 ± 6.5	99 ± 4.7	97 ± 3.7	86 ± 4.7	102 ± 4.0
Pulse	30	36	39	41	34	37
Kidney wt., mg./ 100 cm.^{2}	323 ± 5.2	362 ± 12.2	367 ± 10.1	266 ± 15.8	254 ± 4.9	275 ± 11.5
Heart wt., mg./ 100 cm.^{2}	187 ± 4.2	186 ± 3.7	204 ± 10.5	179 ± 4.3	186 ± 6.4	191 ± 8.5
Adrenal wt., mg.	26 ± 1.1	12 ± 1.4	11 ± 0.8	25 ± 2.1	24 ± 1.6	14 ± 1.6

$$\epsilon = \sqrt{\frac{\Sigma x^2 - [\bar{x}(\Sigma x)]}{n(n-1)}}$$

While Compound F did tend to inhibit body growth, this was not so dramatic as in previous experiments where larger doses had been used. Surprisingly, neither the addition of saline nor previous uninephrectomy appeared to intensify the growth retardation significantly, although this point is not clear cut. The steroid's effectiveness was, however, indicated

by the reduction in adrenal size, although again this effect was not heightened by the intensifying procedures.

No significant change occurred in plasma sodium levels; the apparent increase in the Compound F treated intact animals being negated by the apparent fall in the uninephrectomized group. Similarly, no significant change occurred in plasma potassium levels. In this latter connection, we call attention, however, to the fact that a slight increase in plasma potassium did occur in the groups receiving Compound F. As will become apparent in other experiments, this may represent a subtle change in electrolyte balance.

The effect of Compound F on blood pressure, while statistically significant, is not as dramatic as that previously reported. This was to be expected in the intact animals, since not only was a small dose used, but also larger animals. On the other hand, procedures which are effective intensifiers of the cardiovascular effects of DCA were certainly not operative in Compound F treated animals. The only exception to this statement, which holds both for our blood pressure findings and organ weights is the greater heart weight in those Compount F treated animals which received saline.

Renal function, if kidney weight is any indication (Friedman and Friedman, 1949) is not much affected by the steroid under any of the circumstances noted here. No histological changes were noted in the kidney.

All in all, this experiment seems to indicate that Compound F must act rather differently to DCA and certainly without as much kidney involvement.

Experiment 2. The effect of bilateral nephrectomy on the blood pressure response to Compound F

This experiment was designed to follow up the suggestion of the previous work that the rise in blood pressure in Compound F treated rats was not mediated by the kidney. Thirty-two male albino rats averaging 140 grams in weight were divided into two groups. Group 1 received no treatment while group 2 received a daily injection of 1 mg. of Compound F subcutaneously for 31 days. This small dose was selected as one which would not complicate the experiment by producing marked body weight changes, although, to be sure, neither could it be expected to do much in the way of elevating blood pressure. Table 2 presents the findings.

As expected, the dose of Compound F used did not markedly interfere with growth and the animals were in good condition for the procedure. As expected also, blood pressure was minimally, although significantly, elevated. It should, of course, be noted that femoral arterial pressures show less excursion of the pressure wave and are accordingly not compar-

TABLE 2

Group	Untreated		Compound F	
Initial body wt., gms.	138 ± 4.6		142 ± 9.1	
Final body wt., gms.	278 ± 5.0		221 ± 7.0	
Direct Femoral Blood Pressure				
Systolic	105 ± 3.1		115 ± 4.8	
Diastolic	82 ± 2.8		91 ± 3.5	
Mean	94 ± 5.1		102 ± 4.2	
	Intact	Bilateral nephrectomy	Intact	Bilateral nephrectomy
Direct Aortic Blood Pressure 2 Days Later				
Systolic	119 ± 4.4	129 ± 2.4	132 ± 4.3	133 ± 1.6
Diastolic	76 ± 3.0	91 ± 2.1	85 ± 3.6	82 ± 5.1
Mean	95 ± 2.3	109 ± 3.6	106 ± 1.8	105 ± 4.7

able to aortic pressures. Bilateral nephrectomy did not cause a fall in blood pressure in Compound F treated animals.

Although this experiment, particularly considered together with the first, reaffirms the suggestion that the effect of Compound F is independent of the kidney, it should be borne in mind that a nephrectomy experiment of this type is open to criticism. Accordingly, we would not go further at this point than to suggest that the failure of intensifying measures and nephrectomy to influence the blood pressure rise caused by Compound F, as well as the absence of histological evidence of renal damage in Compound F treated rats, indicates that the kidney is not directly involved.

Experiment 3. The relative susceptibility of young and older rats to Compound F

It had been assumed in arranging the previous experiments that there was a difference in the relative susceptibility of younger and older rats to the blood pressure raising effect of Compound F. This was based on an impression from some of our preliminary work with Compound F. It seemed to us now of some importance to determine whether there was any validity to this impression.

Twenty-four male albino rats of varying weights were arranged in three groups. Group 1, averaging 73 grams in weight, served as untreated controls. Group 2, averaging 81 grams in weight, served as the sample of young rats to receive Compound F and group 3, averaging 242 grams, served as the sample of older rats to receive the steroid. The young rats were given 1.5 mg. daily of Compund F while the older rats were given 3 mg. daily. Treatment was carried on for 23 days. Table 3 presents the pertinent data.

It is apparent that it is easier to elevate the blood pressure with Com-

TABLE 3

Group	Control	Young rats, compound F	Older rats, compound F
Initial body wt., gms.	73 ± 2.7	81 ± 4.1	242 ± 1.2
Final body wt., gms.	173 ± 5.0	94 ± 6.3	206 ± 8.0
Direct Aortic Blood Pressure			
Systolic	121 ± 2.3	146 ± 5.2	134 ± 5.6
Diastolic	72 ± 3.4	85 ± 7.9	85 ± 6.3
Mean	90 ± 3.4	108 ± 8.7	102 ± 3.5
Heart wt., mg./100 cm.²	260 ± 9.6	284 ± 8.6	265 ± 16.1
Kidney wt., mg/100 cm.²	542 ± 8.5	614 ± 22.2	577 ± 14.9

pound F in young rats; while older rats do respond with an elevation of blood pressure they require a larger dose.

Experiment 4. The effect of Compound F in adrenalectomized animals

In view of the considerable amount of evidence suggesting that the adrenal is concerned with some activity in opposition to hypertensive agents such as DCA (Friedman and Friedman, 1951; Pines *et al.*, 1948) it seemed important to determine the effectiveness of Compound F in the adrenalectomized rat.

Twenty-four male albino rats weighing over 200 grams were divided into 3 groups. Group 1 served as untreated controls; group 2 received 2 mg. of Compound F acetate daily for 27 days by the usual route. As has been shown, this amount of steroid in such older rats will ordinarily not elevate the blood pressure to any significant extent. The animals of group 3 were bilaterally adrenalectomized at the start of the experiment and, like group 2, received 2 mg. of Compound F daily. Table 4 presents the data.

TABLE 4

Group	Control	Compound F	Adrenalectomy +compound F
Initial body wt., gms.	230 ± 4.9	248 ± 14.3	263 ± 6.9
Final body wt., gms.	290 ± 6.8	234 ± 6.8	213 ± 8.6
Direct Aortic Blood Pressure			
Systolic	117 ± 4.5	122 ± 6.8	138 ± 3.8
Diastolic	78 ± 2.3	79 ± 7.0	97 ± 2.7
Mean	92 ± 11.0	92 ± 8.1	112 ± 3.3
Kidney wt., mg./100 cm.²	394 ± 14.0	447 ± 20.0	453 ± 20.0
Plasma Na mEq	150.6 ± 3.4	147.8 ± 4.3	150.0 ± 1.1
Plasma K mEq	4.0 ± 0.2	4.2 ± 0.2	4.7 ± 0.2

It is clear that Compound F in the dose given and in the older rats used here did not elevate the blood pressure in intact animals. In marked and clear cut contrast, a significant hypertension was produced by the same dose in the absence of the adrenals. Noteworthy also is the recurrence

of the theme that the hypertension is independent of any significant kidney derangement as indicated by kidney weight.

It should also be pointed out at this time that while in no experiment was there ever a significant change in plasma electrolyte levels, there was usually a moderate elevation in the potassium levels. The recurrent nature of this finding suggests that some modification of the electrolyte pattern is an associated phenomenon in Compound F treated animals.

DISCUSSION

The present studies concerning the blood pressure raising effect of Compound F amplify our previous observations. The interesting fact that the blood pressure rise is not potentiated by those measures which ordinarily increase the effectiveness of DCA fits well with the observations of Knowlton *et al.* (1952) that cortisone produces a hypertensive response more readily in the absence of dietary sodium. These findings suggest a difference in the modes of action of DCA and the naturally occurring adrenal steroids. While in general the observation that natural adrenal cortical hormones can elevate the blood pressure lends substance to much of the inferential work with DCA hypertension, the marked differences here noted make caution in interpretation necessary.

The experiments here reported also call attention to some difference in susceptibility between young and older rats to the cardiovascular effects of Compound F. The effectiveness of this steroid in the adrenalectomized animal raises again the suggestion that among the already numerous possible causes for hypertensive vascular disease should be numbered a failure of the adrenal to inhibit certain pressor agents.

It is also apparent from the present experiments that the kidney does not seem to play any significant primary rôle in the hypertensive response to Compound F. Experiments in progress indicate that the steroid has direct action on the vascular bed in contrast to DCA. More investigation is required, however, before any interpretation can safely be attempted.

SUMMARY

Compound F acetate was administered to rats in experiments designed to test for similarities with the known cardiovascular-renal effects of DCA.

The blood pressure raising effect of Compound F is not intensified, like that of DCA, by uninephrectomy or the substitution of 1% saline for drinking water, either alone or in combination.

The elevation in blood pressure caused by the steroid is not abolished by complete nephrectomy.

Young rats are considerably more sensitive than older animals to the blood pressure raising effect of Compound F.

The increased resistance of older rats is largely abolished by prior adrenalectomy.

Acknowledgment

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PURIFICATION OF BOVINE THYROTROPIC HORMONE BY ION EXCHANGE CHROMATOGRAPHY[1]

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PREVIOUS chemical work directed toward isolation of the thyrotropic hormone from the anterior pituitary (usually bovine) has almost always depended upon precipitation methods of purification (Janssen and Loeser, 1931; Junkmann and Schoeller, 1932; Loeser, 1932; Greep, 1935; Lambie and Trikojus, 1937; Bonsnes and White, 1940; Fevold *et al.*, 1940; Fraenkel-Conrat *et al.*, 1940; Ciereszko, 1945). A number of reagents used in the fractionation of various proteins have been applied to crude extracts; thyrotropin has been found to be soluble in dilute but precipitated in concentrated aqueous solutions of common organic solvents (Janssen and Loeser, 1931; Junkmann and Schoeller, 1932; Loeser, 1932; Bonsnes and White, 1940; Fraenkel-Conrat *et al.*, 1940; Ciereszko, 1945) and salts (Fevold *et al.*, 1940; Fraenkel-Conrat *et al.*, 1940), and it has been precipitated by various organic acids, heavy metal salts, and other protein precipitants (Loeser, 1932). Lack of both specificity and selectivity is the essential limitation of these methods applied to thyrotropic hormone. Thus, they must be repeated and combined (Ciereszko, 1945; Fraenkel-Conrat *et al.*, 1940) and yield must be sacrificed in order to obtain much purification. Furthermore, the solubility behavior varies with the preparation under study, in particular with respect to its purity. This fact suggests the primary role of coprecipitation in the observed precipitation reactions of thyrotropic hormone.

Early reference to the importance of adsorption phenomena was made by Junkmann and Schoeller (1932), who noted adsorption of thyrotropin on various colloids and precipitates, and by Loeser (1932), who commented that the numerous reagents which precipitated thyrotropin from crude extracts might act by coprecipitation. Both Greep (1935) and Lambie and Trikojus (1937) removed thyrotropic hormone from solution by adsorption on precipitated benzoic acid. Jorgensen and Wade (1941) emphasized the great losses by previous procedures and consolidated evidence on coprecipitation, reporting that flavianic, picric, phosphotungstic,

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trichloroacetic, and sulfosalicylic acids precipitated 60 to 90% of the activity from crude extracts but little or none from more purified preparations. These workers found that many commonly used adsorbents picked up thyrotropin but elution was difficult, as found by Junkmann and Schoeller (1932) with precipitates. However, adsorption on Folin Decalso at pH 4.5 by the batch technique and elution with NaOH solutions gave 80 to 100% recovery of activity. No data with regard to degree of purification were presented by Jorgensen and Wade; in the light of the findings to be presented here (*vide infra*), it is likely that only very limited purification was accomplished. The present paper is a report of the application of the more efficient technique of column chromatography, particularly with synthetic ion exchange resins, to the purification of bovine thyrotropic hormone.

EXPERIMENTAL

The assay of thyrotropic activity was based on the thyroid weight increase of day-old White Leghorn male chicks (Bergman and Turner, 1939) given three daily 0.5 ml. subcutaneous injections and autopsied on the fourth day. This procedure resulted in a somewhat less sensitive assay than the original five-daily-injection method of Smelser (1938). Screening work was conducted with groups of 5 to 6 chicks; larger groups of 8 to 11 were used for some confirmatory studies. In this article the dose producing a minimal statistically significant mean thyroid weight increase over controls, under the conditions used, will be abbreviated as one m.s.d.

Data were obtained by directly comparative assays, i.e., in each assay two or three dose levels of a "standard," which was the starting material of the purification procedure, and one or two dose levels of each unknown were administered. Calculations included only responses significantly ($P < 0.02$) higher than control values and hence valid. Only the lower range of thyroid weight response (as recommended by Smelser, 1938) was included, up to a mean thyroid weight double that of controls. In that range, preliminary assays indicated that curves of mean thyroid weight vs. log dose were linear as a first approximation and that crude and purified preparations gave roughly the same slope. Unknown figures were thus determined by direct interpolation on the standard curve obtained in the same assay. Actually, in most cases the response to a dose of unknown fell very closely on one of the standard responses. Table 1 presents an illustrative assay, including duplicate standard determinations. Generally, with groups of 5 to 6 chicks these assays readily differentiated between a given dose and one-half and two times that dose.

Details of the procedure used were as follows: chicks[2] received by mail

[2] From the Hall Brothers Hatchery, Wallingford, Connecticut.

TABLE 1. A TYPICAL ASSAY

Group	Number of chicks	Dose	Mean thyroid weight in mg.	$10^6 \times \frac{\text{mean thyroid weight}}{\text{Body weight}}$
A	8	Control	3.04 ± 0.19	81 ± 4.9
B	5	0.23 mg. T15*	3.48 ± 0.41	92 ± 9.7
C	5	0.45 mg. T15*	4.30 ± 0.33	118 ± 7.3
D	5	0.25 mg. T15	3.49 ± 0.25	95 ± 6.3
E	5	0.50 mg. T15	4.58 ± 0.44	121 ± 13.2
F	5	1% of total 1*M* NaCl eluate from small IRC-50 column run with 45 mg. T15.†	4.30 ± 0.33	120 ± 11.5

Each mean value is followed by its standard error (standard deviation of the mean).

* This set of standards was made up independently and kept in the refrigerator (0–5° C.) for one week prior to beginning of injections.

† Activity from 45 mg. T15 was adsorbed to the buffered Amberlite IRC-50 column and eluted with 25 ml. 1 *M* NaCl, followed by H_2O to final volume of 75 ml., which was then diluted 1:2 for assay.

24 hours after hatching were marked with dyes and all kept in the same electrically-heated commercial starter brooder[3] in a room maintained at about 27° C. and free of drafts.[4] They were fed water and mash of half ground wheat, half ground corn.[5] After three daily subcutaneous injections, on the fourth day chicks were killed in an ether chamber, and the thyroids were dissected out from a cephalad approach, trimmed on moist Whatman #50 filter paper in a Petri dish under the ten power stereoscopic microscope, dried by rolling on similar paper, and weighed immediately on a torsion balance of 25 mg. capacity and 0.01 mg. sensitivity. Assay solutions were kept in the refrigerator (0–5° C.), since no difference in potency could be detected under these conditions as compared with frozen storage.

Estimation of Purification: In crude fractions the estimation of specific activity (potency) was based on the dry weight of water-dialyzed product after lyophilization. For the more purified preparations this method was unsuitable because of the low net weight relative to that of the container, although rough confirmation of the order of magnitude of purification indicated by ultra-violet spectrophotometry (*v.i.*) was found in the case of Amberlite IRC-50 chromatography.

Therefore, concentration of protein-like[6] material was followed by meas-

[3] Sears, Roebuck, & Co.'s starter brooder.

[4] Despite these conditions, during especially cold weather there occurred an unpredictable increase in variability of both control thyroid weight and sensitivity to thyrotropic stimulation, presumably in relation to chilling of the chicks in transit via parcel post.

[5] Use of commercial starter mash, containing fish meal, resulted in larger control thyroid weights, more variable thyroid weights, and less sensitive response to thyrotropin.

uring the absorption at 280 mμ in the Beckman DU spectrophotometer. Although this material was probably chiefly protein, it will be denoted as u.v.a.m. (ultra-violet absorbing material) to avoid the implication that its chemical nature has been characterized fully. Spectrophotometry at 275–280 mμ has been used for determination of the concentration of a pure protein in solution with error of only 1–2% (Univ. Lab. Phys. Chem., Harvard University, 1951), but the components of a crude protein mixture would show considerable differences in coefficients of extinction.[7] With the progress of fractionation, homogeneity increases and the correlation between u.v.a.m. and dry weight should improve, however. In the present work 280 mμ was found to represent a definite absorption peak of the crude thyrotropin; in purified eluates from Amberlite IRC-50 columns (*v.i.*) the maximum was shifted to 275 mμ, but absorption there was only ca. 1% greater than at 280 mμ.

Solutions were kept in the refrigerator until shortly before measurements, which were carried out preferably within a few hours of preparation; even in the refrigerator, solutions tended to develop fine turbidity slowly with time. Optical density plotted against concentration resulted in a straight line.

pH Measurements were made in the Cambridge "research" model glass electrode pH meter.

Preparation of crude material from 1 kg. whole frozen bovine pituitaries primarily followed the first steps of Ciereszko (1945), except that H_2O was found as effective as 1% NaCl for extraction. To the pH 4.1 supernatant was then added, with continuous stirring, enough ammonium sulfate to result in 3.6 *M* (0.9 saturated) concentration. After settling overnight in the cold (4–9° C.), the precipitate was recovered by centrifugation or by filtration through a large sintered glass funnel and was transferred with water to Visking cellulose tubing (medium thickness) for dialysis with slow continuous flow of cold (0–5° C.) distilled water until the effluent was sulfate-free. After centrifugation, the precipitate, which gave no thyrotropic response at the dose of 1 mg. dry weight, was discarded. Upon lyophilization, the supernatant yielded 12–13 grams of water-soluble "crude thyrotropin," with a m.s.d. of ca. 0.2 mg.

CHROMATOGRAPHIC PURIFICATION

Technique: Analytical grade adsorbents and reagents were used. In all cases, the adsorbent was supported on a pad of glass wool in a cylindrical

[6] It was precipitated in 3.6 M $(NH_4)_2SO_4$ and retained by a dialysis membrane (thin or medium Visking cellulose tubing).

[7] Apparently primarily due to differences in aromatic amino acid content (Crammer and Neuberger, 1943).

"Pyrex" tube equipped with an outflow stopcock and was poured from water slurry and packed with rotation and tapping of the tube during settling by gravity. The solution was admitted automatically at the outflow rate from a separatory funnel in a stopper plugged into the top of the chromatography tube. To avoid disturbing the surface of the bed, at least 1 to 2 cm. of liquid were maintained above it, except when the influent

TABLE 2. BEHAVIOR OF ADSORBENTS WITH CRUDE THYROTROPIN

Adsorbent	Effluent pH	Thyrotropic activity		Adsorption of inactive material (u.v.a.m.)
		Adsorption	Elution	
Amberlite IRC-50	4 (H-form) 7–8	0 Quantitative (>97%)	— Easy, complete	High Low
Folin Decalso ("permutit")	4–8	Quantitative (>90%)	Fairly easy, probably less complete than with IRC-50	High (but much was left on column by neutral eluents)
Amberlite IR-4B	≦4 (HCl-form) ≧8 (basic form)*	0 0	— —	Low Very low
Amberlite XE-67	4–6 (Cl-form) Partial monobasic and dibasic phosphate and partial OH-forms†	0 0	— —	Very low Low
Solka-Floc BW-200	4.8–6.2	Quantitative (>80%)	Unsuccessful	Not measured‡

Elution was by 1 *M* NaCl, $CaCl_2$ or HCl except in the case of Folin Decalso, which did not receive HCl.

* Influent pH's 4–9.9.

† Influent pH's all 4.1, effluent pH's not measured.

‡ But probably high—effluent was colorless, despite dark brown influent.

solutions were changed. At this time the liquid level was brought down to the adsorbent surface and brought up again carefully with the new influent.

Screening studies were carried out with 45–50 mg. crude thyrotropin in 15 ml. on columns 1.5 cm. in diameter and 13 to 16 cm. in final length, using flow rates of ⅔ to 1 ml. per minute. In purification experiments 450–700 mg. crude thyrotropin in 90–150 ml. solution were passed through columns of 2.7 to 2.8 cm. diameter and 27 to 30 cm. final length. Flow rates were 1.2 to 1.6 ml. per minute. Work was carried out at room temperature without appreciable loss of activity. However, solutions were kept in the refrigerator (0–5° C.) except during the actual procedure.

Choice of Adsorbents: Results of screening experiments are summarized in Table 2. Further work for the most part was concentrated on Amberlite IRC-50, but brief consideration will also be given to the other adsorbents.

TABLE 3. ACTIVITY BREAKTHROUGH: AMBERLITE IRC-50 COLUMNS PASSED BY CRUDE THYROTROPIN SOLUTIONS

	pH 4 (H-form)	pH 7.4–8.0	pH 8.2	pH 8.8	pH 9.2–9.3
Activity Leakage	100%	0(<2.5%)*	ca. 5%*	≧10%	≦30%*

* Average of two or more runs.

Amberlite IRC-50[8] is a synthetic carboxylic cation exchange resin in the form of spherical granules, mostly in the size 20–50 mesh. A weak acid, it has little tendency to exchange except by neutralization with bases, but its salt form exchanges cations easily. By partial neutralization with base and the passage of a buffer solution of appropriate pH through the column, to distribute the partial neutralization evenly over the adsorbent, a resin bed of desired pH and high buffer capacity is obtained. The resin swells as much as 100 per cent in complete neutralization. One-fourth (0.25) *N* NaOH and 0.5 *M* sodium phosphate buffer of desired pH were used to obtain the partial sodium form in these experiments. No adjustment of the pH of the crude thyrotropin solution was necessary before passage.

Table 3 summarizes the results of thyrotropic assay of the percolate of columns at various pH's. Extensive development of the column with distilled water after the adsorption run carried off no detectable activity early or late in the washing. Even at a pH above 9, no further activity was carried off by water washing subsequent to the adsorption run itself. In contrast to the quantitative adsorption of activity, ca. 80% or more of the influent u.v.a.m. breaks through at pH 8, 65–80% at pH 7. Of this, about 10–20% is contributed by development of the column with H_2O.

Table 4 summarizes an experiment designed to determine the capacity for adsorption of the activity from crude thyrotropin by a small pH 8.0 IRC-50 column (1.5 cm. in diameter, 9.0 cm. long in H-form, 15.5 cm. long

TABLE 4. CAPACITY OF SMALL AMBERLITE IRC-50 COLUMN AT PH 8.0 FOR ACTIVITY FROM CRUDE THYROTROPIN SOLUTION

	Influent	Effluent: 20 ml. fractions				
		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Activity in m.s.d./ml.	ca. 27	0	0	$<\frac{2}{3}$	ca. $\frac{2}{3}$	ca. 1 ($\frac{2}{3}$–$1\frac{1}{3}$)

105 ml. of crude thyrotropin solution was passed continuously through a pH 8.0 column of Amberlite IRC-50 (1.5 cm. in diameter and originally 9.0 cm. long in the H-form). After discard of the first 11 ml. (ca. 6 ml. voids), 20 ml. fractions of the effluent were collected and assayed.

[8] Samples were kindly supplied by Rohm and Haas Co., the Resinous Products Division, Philadelphia 5, Pennsylvania.

after preparation) representing 15.8 ml. of packed H-form of the resin. There was no sharp, complete breakthrough of activity. Over 1080 m.s.d. were adsorbed without loss, 1620 additional m.s.d. with only 2.5 to 5% loss. Thus, a total capacity of over 170 m.s.d. per ml. packed H-resin was indicated.

This finding was confirmed in another experiment which gave a value of over 170 m.s.d. per ml. packed H-resin with 5 to 10% leakage. In actual use for purification only 23–35 m.s.d. per ml. packed H-resin were applied to the column.

Adsorption of thyrotropic hormone directly from the undialyzed crude extract or pH 4.1 supernatant was not satisfactory.

Elution of the thyrotropic activity by 1.00 *M* NaCl from columns of Amberlite IRC-50 was found to be complete, within the inherent limits of

TABLE 5. PURIFICATION OF CRUDE THYROTROPIN ON 2.7 CM.–DIAMETER AMBERLITE IRC-50 COLUMNS

	Volume (ml.)		Activity (m.s.d./ml.)		Optical density at 280 mμ vs. H_2O		Relative potency	
	A‡	B§	A	B	A	B	A	B
Influent solution of crude thyrotropin	140	140	13	13	4.61	4.61	1	1
Breakthrough:								
Fraction 2*	70	70	0	0.7	2.96	3.62	negligible	0.07
Fraction 3†	50	50	0	0.7	3.15	3.66	negligible	0.07
Eluate:								
Fraction 1	129	155	11	12	0.256	0.255	15	17
Fraction 2	50	50	6	6	0.088	0.097	24	22
Fraction 3	50	30	0.7	0.7	0.063	0.079	4	3

* Breakthrough from 70 ml. after voids until the end of the column input.
† Next 50 ml. of the breakthrough, column driven with H_2O.
‡ A was run on a pH 7.7 column, developed with 150 ml. H_2O, and eluted with 150 ml. of 1*N* HCl, followed by H_2O.
§ B was run on a pH 8.2 column, developed with 150 ml. H_2O, and eluted with 200 ml. of 1 *M* NaCl, followed by H_2O.

accuracy of the biological assay. Examples are found in Tables 1 and 5. Subsequent parallel elution experiments showed that equimolar $CaCl_2$ and HCl solutions eluted the same amount of thyrotropic activity as 1.00 *M* NaCl and that similar volumes of these three solutions were needed for this action. "Crossing over" the eluents after all activity apparently had been eluted yielded no further activity, and ascending concentrations (0.125 *M* up to 4.00 *M*) of NaCl eluted the same total activity as 1.00 *M* NaCl. These facts were considered to adduce support for the presumption of quantitative recovery of activity, in view of the marked difference of affinity of H^+, Ca^{++}, and Na^+ for neutralized carboxylic resins.

Typical results with the final recommended chromatographic procedure

(*v.i.*) are shown in Table 5. A 14- to 16-fold purification was accomplished with excellent recovery of activity. If pH 7.0 columns were used similarly, ca. 10 (9–11) times purification resulted. In one experiment with medium-sized pH 7.0 columns, elution with successive 50 ml. portions of 0.125, 0.25, 0.50, 1.00, 2.00, and 4.00 *M* NaCl, with mixing above the column upon entry, produced purification ca. one-third greater than that with 1.00 *M* NaCl.

Attempts to repurify NaCl or HCl eluates directly on a second IRC-50 column were unsuccessful. Leakage of activity greater than 20% was found early in the percolate and rose rapidly to 100%; capacity for adsorption of thyrotropic hormone from these eluates was thus exceedingly low. This may be explained by their high (non-protein) buffering capacity and solid (salt) content.

Folin Decalso,[9] "permutit according to Folin," is a synthetic aluminosilicate cation exchange material ground to 60–80 mesh. With effluent pH's 4 to 8, over 90% adsorption of thyrotropic activity was obtained on such columns. Only 10–20% of the influent u.v.a.m. was found in the percolate, even after development with water twice the volume of the crude thyrotropin solution.

The major portion of the activity was eluted by 1.00 *M* $CaCl_2$ or NaCl (see Table 2). With an equal volume of 0.50 *M* Na_2HPO_4 somewhat less activity was recovered and more u.v.a.m. was eluted than by 1 *M* NaCl, while with 0.25 *M* NaOH less than 25% of the activity was eluted but over twice as much u.v.a.m. as with 1 *M* $CaCl_2$ or NaCl. Thus it is evident that alkaline solutions elute an excessive amount of inactive material. The purification achieved with the aid of 1.00 *M* $CaCl_2$ or NaCl elution from small columns run at a pH of ca. 7 was only 4 to 6-fold if recovery was assumed to be quantitative. This is no better than half the purification obtained by the similar use of small columns of Amberlite IRC-50.

Folin Decalso columns with effluent pH's 6.5–7.7 adsorbed the thyrotropic activity quantitatively (>90%) from NaCl or HCl eluates of Amberlite IRC-50 columns, but attempts to elute it with 1.00 *M* $CaCl_2$, 1.00 *M* NaCl, or 4.00 *M* NaCl were unsuccessful.

Powdered Cellulose: Solka-Floc BW-200[10] columns adsorbed over 80% of the activity from both crude thyrotropin solutions and HCl and NaCl eluates from Amberlite IRC-50 columns. In all cases, 1.00 *M* NaCl, $CaCl_2$, and HCl eluates were inactive at two times the theoretical m.s.d. for complete recovery.

Anion Exchange Resins: Amberlite IR-4B[8] is a coarsely granular, weakly

[9] A sample was generously provided by the Permutit Co., 330 W. 42 St., New York 18, N. Y.

[10] A sample was kindly provided by the Brown Co., Berlin, N. H.

basic resin. It thus has only slight tendency to exchange except by neutralization with moderately strong acids. Both hydrochloride and base forms permitted quantitative passage of thyrotropic activity into the percolate (see Table 2). U.v.a.m. breakthrough was also high: ca. 90% by the base, ca. 67% by the hydrochloride form. Hence it is of little use for purification of thyrotropin.

Amberlite XE-67[8] is a more finely divided, strongly basic resin, supplied in the neutralized chloride form. Quantitative breakthrough of thyrotropic activity occurred on passage of pH 4.1 solutions of crude thyrotropin through columns of the chloride form or chloride form partly converted to the $H_2PO_4^-$, $HPO_4^=$, or OH^- form. Another experiment with effluent pH of 6.1 confirmed quantitative passage of the chloride form. U.v.a.m. breakthrough was 70–80% for the Cl-form, ca. 55% for the partial phosphate and hydroxyl forms.

PRECIPITATION REACTIONS

Technique: Cold NaCl or HCl eluates, from Amberlite IRC-50 columns of pH 7.0, with a purity ca. 10 times that of crude thyrotropin, were treated with a number of common protein precipitants. Reagent solutions were added dropwise with continuous stirring, trichloroacetic acid in 20 gm. per 100 ml. concentration to the calculated amount, other reagents as saturated aqueous solutions until precipitation appeared complete. After standing overnight in the refrigerator, the suspension was centrifuged 30 minutes at 3000 r.p.m. Trichloroacetic acid precipitates were washed with a small volume of the final concentration of trichloroacetic acid and recentrifuged; other precipitates were not washed.

Results are summarized in Table 6.

The phosphotungstic acid supernatant lost its activity after treatment with slightly excess saturated $Ba(OH)_2$, centrifugation, neutralization with 0.2 *M* H_2SO_4, and recentrifugation.

The acid was removed from the supernatants from the picric and flavianic acid precipitations by passage through columns of the base form of Amberlite IR-4B without loss of their thyrotropic activity.

By precipitation of impurities, picric acid treatment of an eluate from a pH 7 Amberlite IRC-50 column accomplished ca. 2 times further purification, and trichloroacetic acid to 5 gm. per 100 ml. gave 3 times purification of a pH 8 column eluate. These calculations are based on the optical density at 280 mμ after dialysis against water for 3–4 days in thin Visking cellulose tubing. The recovery of activity after dialysis was definitely more than 70 per cent; under the conditions of assay this was indistinguishable from quantitative recovery (*v.s.*).

An active picric acid supernatant prepared from IRC-50 eluates treated

TABLE 6. PRECIPITATION REACTIONS OF ELUATES FROM pH 7 AMBERLITE IRC-50 COLUMNS

Treatment	% Total Thyrotropic Activity	
	Supernatant	Precipitate*
Picric Acid†	100%	0 (<10%)
Trichloroacetic Acid:‡		
final conc. 0–2.5 gm./100 ml.	Not assayed	0 (<10%)
2.5–5	Not assayed	0 (<10%)
successive 5–10	Not assayed	ca. 30% (>15, <50)
0–5	ca. 100%	0 (<10%)
0–10	Not assayed	ca. 15%
Phosphotungstic Acid†	ca. 80% (≫50, ≦100)	ca. 15% (9–18)
Flavianic Acid†	ca. 60% (>50, ≪100)	ca. 30% (≧20, <50)

* Precipitates were not washed, except for the trichloroacetic acid ppts., which were washed in a small volume of the final concentration of trichloroacetic acid and recentrifuged.
† Added as saturated aqueous solutions until precipitation was complete.
‡ Added as 20 gm. per 100 ml. solution to the calculated final concentration.

with ethanol to a final concentration of 85 volumes per cent yielded a precipitate inactive at 4 times the m.s.d. theoretical for complete recovery. Similarly, 1/15 the volume of saturated alcoholic benzoic acid added to a picric acid supernatant left activity in the supernatant and the voluminous precipitate was inactive at a dose of 5 times the theoretical m.s.d.

RECOMMENDED PURIFICATION PROCEDURE

Crude thyrotropin is prepared by the method described in an earlier paragraph.

A column of Amberlite IRC-50 2.7 cm. in diameter and 15–16 cm. long is treated with successive portions of 500–600 ml. 0.25 *N* NaOH, 800 ml. H_2O, 250 ml. 0.5 *M* sodium phosphate buffer of pH 8.0, and 800 ml. H_2O. 100–140 ml. of a solution of 5.0 mg. crude thyrotropin per ml. is passed through the column, followed by 150 ml. or more H_2O (until the optical density of the effluent at 280 mμ in the Beckman DU is less than 0.100). 200 ml. 1.00 *M* NaCl (or HCl or $CaCl_2$) is then passed through, followed by H_2O, and the eluate is collected fractionally.[11] After assay, inactive fractions are discarded.

To the cold active eluate, saturated aqueous picric acid is added dropwise until precipitation is complete, or, as an alternative, ⅓ the volume of 20 gm./100 ml. trichloroacetic acid is added dropwise with stirring. After settling overnight, the precipitate is removed by centrifugation, leaving the active supernatant.

Recovery of the dry product from this supernatant may be accomplished

[11] Larger amounts of crude thyrotropin may be purified by this chromatographic procedure with proportionally increased amounts of resin and reagents.

by lyophilization after dialysis, or direct further treatment of the solution could be preceded by acid removal by passage through a column of Amberlite IR-4B.

DISCUSSION

No important innovations were made in choice of starting material, extraction (Loeb and Bassett, 1930; Junkmann and Schoeller, 1932; Loeser, 1932; Chance *et al.*, 1939; etc.) or preparation of crude material. However, the simple and practical chromatographic purification on Amberlite IRC-50 results in a good yield and is the most selective single operation for purifying crude bovine thyrotropic preparations yet reported. The calculated potency of the product is greater than or at least equal to that obtained by Fraenkel-Conrat *et al.* (1940) by repeated acetone and ammonium sulfate fractionation, which resulted in a m.s.d. of 0.01–0.02 mg. by the five-daily-injection chick thyroid weight assay. The simple additional precipitation step described above brings the calculated m.s.d. of my product to ca. 0.004 to 0.007 mg. by the less sensitive three-daily-injection thyroid weight assay. Ciereszko (1945) has reported that by a combination of numerous precipitation methods he achieved good yield of a product with 0.001 mg. m.s.d. by the five-daily-injection chick thyroid histological assay. Recently, there has been a preliminary report by Steelman *et al.* (1952), without details of preparation, of a product with m.s.d. of 0.002 mg. by the chick thyroid iodine depletion (3-day) assay, a method considerably more sensitive. Columns of Folin Decalso with neutral eluents were far less effective than Amberlite IRC-50 in purification, and use of strong alkali for elution especially reduced purification far further (cf. method of Jorgensen and Wade, 1941).

It is strongly suggested by the data on adsorption vs. pH that Amberlite IRC-50 acts as a true cation exchanger here and that the exchanged substance containing (or adsorbing) the thyrotropic activity has a rather strongly basic group or is cationic as a whole up to pH 8. Consistent with the latter possibility is the complementary complete passage of thyrotropic activity through columns of anion exchange resins in this pH range. It is of interest that electrophoretic studies of the purified preparation of Steelman *et al.* (1952) indicated that the thyrotropic activity always concentrated at pH's above 7 and had an apparent isoelectric point of pH 8 to 8.5. Conclusive knowledge of the ionic behavior of the hormone itself must obviously await preparations that are clearly chemically pure.

It is well known that another parameter of importance in adsorption by ion exchange resins may be the degree of porosity of the resin to ions. This is a function of the resin's structure (especially with regard to cross-

linking) and particle size and the size and shape of the ions. Hence the fact that most protein-like contaminants in the crude thyrotropin pass through an Amberlite IRC-50 column at pH 8, while thyrotropin itself is adsorbed quantitatively, is consonant with the possibility that the thyrotropin molecule is smaller or more easily penetrating. This factor, as well as the likely lesser basicity of most of the proteins, as compared with thyrotropin, might play a role in the purification of crude thyrotropin obtained with Amberlite IRC-50.

No specific precipitant for thyrotropin was found in this work, and reactions of purified eluates generally confirmed the precipitation findings of Jorgensen and Wade and were in disagreement with the report of Ciereszko (1945) that picric acid and phosphotungstic acid precipitate thyrotropic hormone completely from highly purified preparations. However, differences in conditions included a high salt content and high buffer capacity (at least against base) of the IRC-50 eluates to which these acids were added; nevertheless, formation of an insoluble picrate or phosphotungstate seems denied. It is noteworthy that activity is not precipitated from picric acid supernatants of IRC-50 eluates even by ethanol to 85 vol.% or by excess freshly precipitated benzoic acid (contrast Junkmann and Schoeller, 1932; Greep, 1935; Lambie and Trikojus, 1937, re cruder preparations). The evident wide differences in solubility behavior of bovine thyrotropic hormone in preparations of different purity support the assumption that precipitation of activity by the reagents used to date has been due primarily to coprecipitation with inactive proteins in the crude extracts. Certainly adsorption of thyrotropin seems widespread. In any case, the present knowledge of solubility behavior is significant only with reference to specific conditions and preparations (Li and Evans, 1948).

A number of workers have reported that bovine thyrotropin in crude preparations does not pass a dialysis membrane or ultrafilter (Junkmann and Schoeller, 1932; Anselmino and Hoffmann, 1934; etc.). Dialysis of my most purified preparation did not indicate gross loss, but the dialysate was not assayed. In view of the extensive adsorption phenomena of thyrotropin, future reevaluation of this question in the most purified preparations would seem to be desirable.

SUMMARY

1. Quantitative adsorption of activity from dialyzed crude bovine thyrotropin preparations was obtained on columns of the carboxylic cation exchange resin Amberlite IRC-50 at pH 7 to 8.0; above this pH, activity leakage appeared progressively. No adsorption to the H-form of IRC-50 was found.

2. Development of the pH 8 column with H_2O and elution with 1.00 *M* NaCl, HCl, or $CaCl_2$ resulted in good recovery of activity and ca. 15 times purification.

3. Treatment of somewhat (ca. $\frac{1}{3}$) less purified IRC-50 eluates with one-third their volume of 20 gm. per 100 ml. trichloroacetic acid solution or with saturated aqueous picric, phosphotungstic, or flavianic acid until precipitation was complete left the majority of thyrotropic activity in the supernatant, quantitatively in the first two cases.

4. A simple recommended procedure for purifying crude thyrotropin, combining chromatography on Amberlite IRC-50 at pH 8 and precipitation of further impurities from the eluate by excess picric acid or trichloroacetic acid to 5 gm. per 100 ml., has been summarized.

5. Columns of Folin Decalso are less than half as effective as Amberlite IRC-50 in purifying crude thyrotropin.

6. Bovine thyrotropic hormone passes columns of the anion exchange resins, Amberlite IR-4B and XE-67, completely at pH's $\leqq 8$.

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DIURESIS DURING MAINTENANCE OF ADRENALECTOMIZED DOGS WITH ASCITES ON CORTISONE

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INTRODUCTION

IT HAS been demonstrated previously (Volwiler, *et al.*, 1950; Hwang, *et al.*, 1950; Davis, Howell and Southworth, 1953) that at least two factors, namely, elevated hepatic venous pressure and adrenocortical salt retaining hormones are essential for sodium (Na) retention during the formation of experimental ascites. The pattern of urinary (low Na and potassium (K)) and fecal (low Na and elevated K) electrolyte excretion characteristic of ascites formation in dogs with intact adrenal cortices (Davis and Howell, 1953) disappeared following bilateral adrenalectomy during maintenance on 0.5 mg./day of desoxycorticosterone acetate (DCA) and ascites ceased to accumulate (Davis, Howell and Southworth, 1953). Subsequently, large doses of DCA effected complete replacement of the abnormal pattern of electrolyte excretion and resulted in rapid accumulation of ascitic fluid. The present study was undertaken to determine whether an 11, 17-oxysteroid would lead to the formation of ascites in adrenalectomized dogs with thoracic inferior caval constriction. Observations were also made during maintenance of simple adrenalectomized dogs[1] on cortisone.

METHODS

The material for this study consisted of two simple adrenalectomized dogs and two adrenalectomized dogs with constriction of the thoracic inferior vena cava; the latter animals were dogs 3 and 4 of a previous report (Davis, Howell and Southworth, 1953). The four dogs were approximately the same size and weight (17–20 kg.).

Control observations were made during maintenance on 3 mg./day of DCA. Replacement therapy was then changed to 20 mg./day of cortisone (Cortone acetate, Merck) and studies made until either 1) electrolyte metabolism reached an apparently stable

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[1] The term "simple adrenalectomized dog" has been used to designate bilaterally adrenalectomized animals with no other alteration.

state in the simple adrenalectomized animals or 2) most of the ascites had disappeared in the adrenalectomized dogs with caval constriction. Subsequently, the dose of cortisone was gradually increased to 65 or 70 mg./day. Recovery was studied during administration of 3 mg./day of DCA.

The dogs were fed a synthetic diet containing 80 Cal./kg./day, 0.2 or 0.6 gm./kg/day of nitrogen, 30 or 60 m. Eq./day of Na and 17.6 m.Eq./day of K. Water was allowed *ad libitum*.

All experimental procedures and chemical methods have been described elsewhere (Davis and coworkers, 1952; Davis and Howell, 1953; Davis, *et al.*, 1953).

RESULTS

Maintenance of Simple Adrenalectomized Dogs on Cortisone

When replacement therapy was changed from 3 mg./day of DCA to 20 mg./day of cortisone, a striking increase in urinary Na excretion occurred (Fig. 1) and urinary K output declined. An accompanying fall in plasma Na and T-1824 dye space and an elevation in plasma K were observed (Fig. 1). Arterial pressure increased and the general appearance and vigor of both animals improved despite the fall in plasma Na. An increase in glomerular filtration rate (GFR) and renal plasma flow (RPF) occurred in dog 1 on 20 mg./day of cortisone whereas no detectable change was observed in dog 2 at this dosage level.

After 20 mg./day of cortisone had been administered for 15 to 20 days, Na and K balances were again achieved although the abnormal pattern of plasma electrolytes remained. Subsequently, the dose of cortisone was gradually increased to 65 or 70 mg./day. Plasma Na and K returned to normal in dog 2 but remained abnormal in dog 1 (Fig. 1). Except for a transient increase in Na excretion, electrolyte balances did not change in either animal. GFR and RPF showed a further slight elevation in dog 1 (Fig. 1); in dog 2, the first discernible increase in renal function occurred during administration of 70 mg./day of cortisone.

During recovery on 3 mg./day of DCA, Na and water were retained and K balance was negative; plasma electrolytes (dog 1) and T-1824 dye space returned to the control level (Fig. 1). GFR and RPF remained at the level observed during cortisone therapy in dog 1 but returned to the control level in dog 2.

Maintenance of Adrenalectomized Dogs with Thoracic Inferior Caval Constriction and Ascites on Cortisone

Observations were first made during administration of 3 mg./day of DCA; 70 to 80% of ingested Na was retained (Fig. 2). Substitution therapy was then changed to 20 mg./day of cortisone. Balances for Na and water became markedly negative, body weight declined and ascites dis-

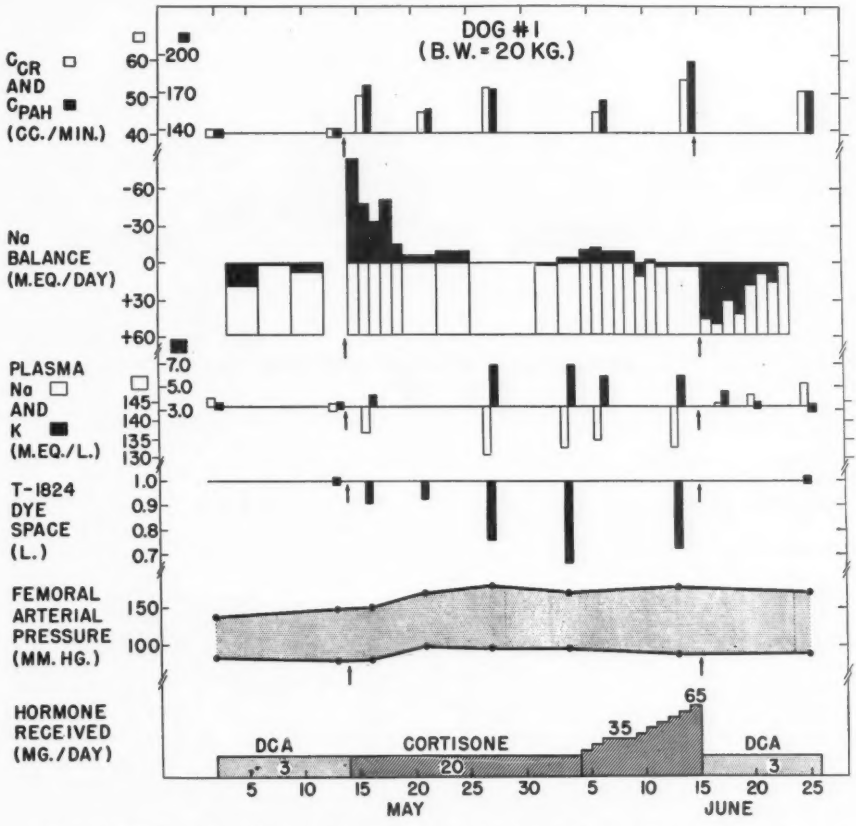

FIG. 1. Efficacy of cortisone for maintenance of a simple adrenalectomized dog. Values for C_{CR}, C_{PAH} and plasma electrolytes are plotted above and below horizontal lines which represent the average of control determinations. The convention for the plot of Na balance is as follows: (1) intake was plotted downward from the zero line, (2) output was plotted upward from intake line, and (3) solid black areas indicate total balance. Femoral arterial pulse pressure is shown by the stippled area. Vertical arrows indicate onset and termination of cortisone administration.

appeared (Fig. 2). Potassium balance was positive at the onset of the diuresis. The normal rate of fecal Na and K excretion observed during DCA administration remained unchanged. Plasma Na fell and plasma K became elevated. GFR and RPF remained unchanged until near the end of the period of administration of 20 mg./day of cortisone at which time an elevation occurred (Fig. 3). The concentration of total plasma protein

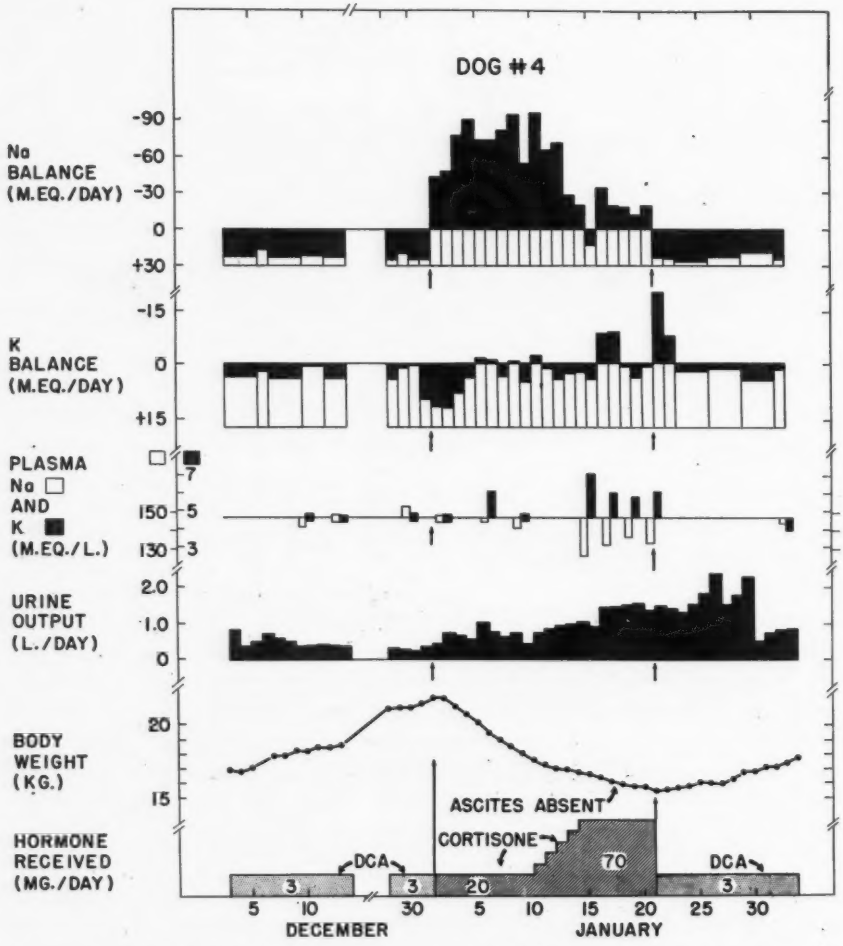

FIG. 2. Failure of cortisone to effect Na retention in an adrenalectomized dog with thoracic inferior vena cava constriction. See Figure 1 for description of symbols.

increased progressively from the onset of the diuresis (Fig. 3). Femoral venous pressure fell as ascites disappeared presumably as a result of a decrease in intra-abdominal pressure; arterial pressure increased slightly.

In an attempt to elicit a salt retaining action of cortisone, the dose was increased from 20 to 70 mg./day and maintained at this high level for 7 days (Fig. 2). Sodium balance continued to be negative, body weight declined further and the concentration of plasma Na and K remained abnormal. GFR and RPF showed a striking increase and arterial pressure

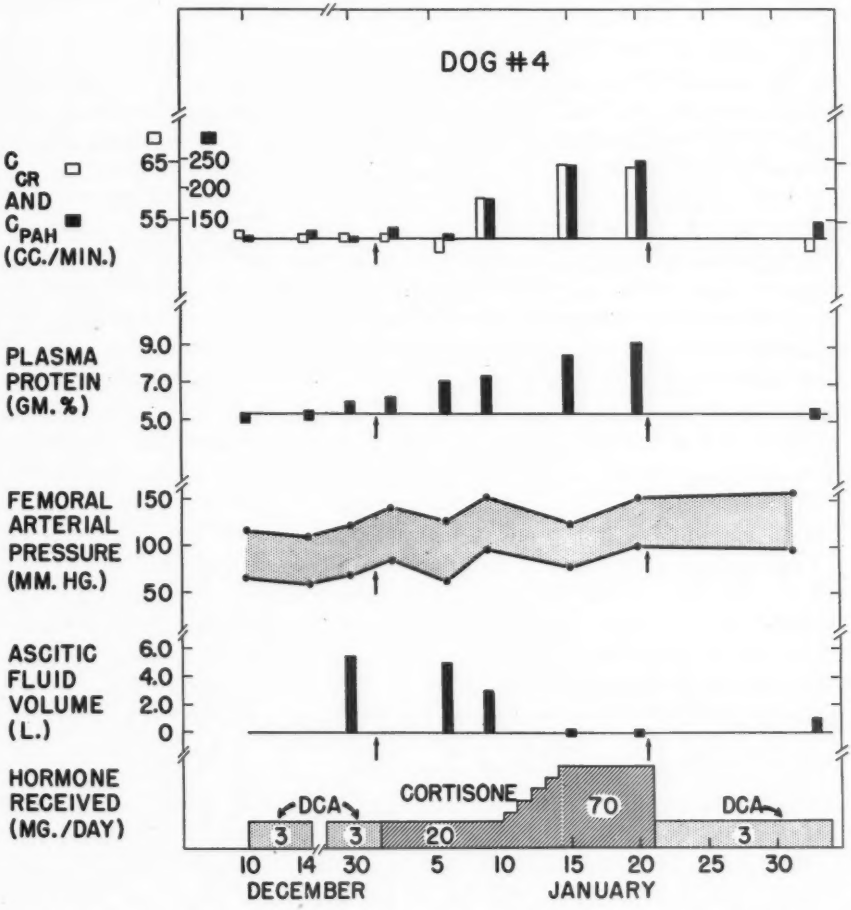

FIG. 3. Renal hemodynamic function, plasma protein, arterial pressure and ascitic fluid volume during maintenance of an adrenalectomized dog with thoracic caval constriction on cortisone. See Figure 1 for symbols.

remained slightly elevated. With the progressive loss of ascitic fluid, the concentration of total plasma protein increased until values of 8.3 and 9.1 gm.% were observed for dogs 3 and 4 respectively.

Subsequent administration of DCA resulted in positive balances for Na and water, gain in body weight and reaccumulation of ascites (Figs. 2 and 3). Renal hemodynamic function and the concentration of plasma Na, K and total protein returned to the precortisone level.

DISCUSSION

The level of renal hemodynamic function which results following the change from DCA to cortisone for maintenance of adrenalectomized dogs is dependent upon several effects. These include 1) a primary action of cortisone on the renal vascular bed, 2) an effect related to contraction of the volume of extracellular fluid and plasma, and 3) an indirect influence on the level of protein metabolism as shown from a previous study (Davis and Howell, 1953) of the effect of protein depletion and repletion on the renal circulation in dogs with experimental ascites. In the simple adrenalectomized dogs, GFR and RPF remained unchanged or increased during administration of 20 mg./day of cortisone in spite of a marked decrease in extracellular and plasma volume (T-1824 dye space). This finding suggests that cortisone exerts a primary effect on the renal vascular bed. Also, an action on the renal vasculature may have occurred in the diuresing adrenalectomized dogs. In addition, the rise in renal function may have been partially related to altered protein metabolism in the dogs with caval constriction; a high concentration of plasma protein occurred during diuresis presumably as a result of mobilized ascitic fluid protein.

The efficacy of cortisone for maintenance of the simple adrenalectomized dog has been described previously by Roberts and Pitts (1952) and by Swingle and associates (1952 and 1953). The present observations provide additional data pertinent to elucidation of the renal mechanisms involved. Since creatinine clearance (C_{CR}) was elevated in dog 1 on 20 mg./day of cortisone and in the other three animals with larger doses, the present results indicate that an elevated GFR contributed to the weak Na retaining effect of cortisone. However, failure of cortisone to effect Na retention in adrenalectomized dogs with caval constriction can not be attributed entirely to elevated GFR; in dogs 3 and 4, Na balance was negative during administration of 20 mg./day of cortisone before C_{CR} was detectably increased. Therefore, Na loss may be related to a weak action of cortisone on Na transport. Potassium retention might also be considered as a manifestation of the inadequacy of cortisone to support the ion exchange process by which K is excreted in exchange for Na (Berliner, 1952).

The most striking finding in the adrenalectomized dogs with caval constriction was the failure of cortisone to produce Na retention at any dosage level employed. Since GFR increased progressively with the larger doses of cortisone and Na balance was negative after all ascites disappeared, it seems unlikely that a dose larger than 70 mg./day of cortisone would have resulted in ascites formation. Smaller doses of cortisone were not employed because Swingle and coworkers (1952) have shown that doses less than 0.9 mg./kg. day are frequently inadequate for maintenance of the simple adrenalectomized dog.

It has been reported previously (Davis and Howell, 1953) that the number of circulating eosinophils is not detectably altered in dogs forming ascites. This finding suggests that the quantity of circulating 11, 17-oxysteroid hormones is not increased in dogs with ascites since these compounds possess the most marked capacity of the adrenal steroids to produce eosinopenia (Thorn, 1953). The present observations with cortisone are consistent with the negative evidence from eosinophil counts and give no indication that 11,17-oxygenated steroids are directly involved in the pathogenesis of ascites. Although this type of evidence does not conclusively exclude an adrenocorticotropic hormone (ACTH) mechanism, it appears that the adrenal cortex is not stimulated excessively by ACTH during ascites formation. In this event, another mechanism may effect increased secretion of adrenocortical salt retaining hormones as postulated previously (Davis, Howell and Southworth, 1953).

SUMMARY AND CONCLUSIONS

Renal function and electrolyte balances were studied during administration of cortisone to adrenalectomized dogs with thoracic inferior caval constriction and to simple adrenalectomized dogs. The increase in GFR and RPF appeared to result primarily from an action of cortisone on the renal vascular bed. Cortisone failed to effect Na retention in the adrenalectomized dogs with thoracic inferior caval constriction. Ascitic fluid accumulated on DCA but diuresis began at the onset of cortisone administration and continued until all ascites disappeared in spite of large doses of cortisone. It is concluded that hormones with DCA-like activity rather than cortisone are essential for ascites formation. In the simple adrenalectomized dogs, Na loss occurred initially but balance was achieved later. The data suggest that cortisone influences Na excretion by two opposing actions 1) augmentation of Na reabsorption by a weak effect on renal tubular transport and 2) increased Na loss by elevated GFR.

Acknowledgements

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MODIFICATION OF THE CHOLINE DEFICIENCY SYNDROME IN THE RAT BY SOMATOTROPHIN AND HYDROCORTISONE[1]

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IMMATURE rats 21–23 days of age and weighing less than 45 gm. characteristically develop degenerative changes and hemorrhagic necrosis of the renal cortex and fatty infiltration of the liver when maintained for a few days on a diet deficient in choline (Griffith and Wade, 1939; Griffith, 1941). The renal changes are only observed to occur in very young rats of the above age and weight, and it seems probable that this is because of the relatively rapid growth which occurs at this period of life. Inasmuch as the 11-oxygenated adrenal steroids exert profound effects on metabolism and upon growth rate in particular it seemed likely that the specific lesions of choline deficiency might be modified by simultaneous treatment with these hormones. It has been found that both cortisone (Sellers *et al.*, 1950) and Upjohn Lipo-Cortex Extract (Hurd *et al.*, 1953) protect rats against the renal changes, and to a lesser extent also against the fat infiltration of the liver. These observations are in accord with data which we have obtained with these preparations, but which were anticipated by the studies cited.

The effects of somatotrophin (STH) on growth and metabolism are in most respects diametrically opposed to those of the 11-oxygenated adrenal steroids and it might reasonably be presumed that the requirement for choline would be increased by simultaneous treatment with this hormone. Indeed, it has been shown recently that pyridoxine deficiency symptoms are enhanced in rats receiving anterior pituitary growth hormone (Beare *et al.*, 1953). The hypothesis was tested by comparing the response of rats subjected to a choline-deficient diet with that of animals also receiving somatotrophin. A group of animals was also treated with Hydrocortisone while on a regimen deficient in choline in order to determine whether this steroid also exhibits the protective effect which has been reported for other adrenal cortical preparations.

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[1] This study was supported by a grant from the American Heart Association.

MATERIALS AND METHODS

Male rats of the Holtzman strain were weaned at 22 or 23 days and fed a purified, choline-free diet, hereafter referred to as basal diet, of the following percentage composition: vitamin-test casein, 20; cystine, 0.3; salts (USP XIII, No. 2) 4; lard, 8; corn oil, 2; celluration, 2; vitamin mixture, 1; sucrose, 62.7. The content of vitamins per 100 gm. diet was: thiamine, 0.5 mg.; riboflavin, 1.0 mg.; pyridoxine HCl, 0.5 mg.; niacin, 2.5 mg.; calcium pantothenate, 5.0 mg.; p-amino-benzoic acid, 1.0 mg.; folic acid, 0.2 mg.; 2-methyl-1,4-naphthoquinone, 0.5 mg.; vitamin B_{12}, 50 μg, biotin, 0.2 mg.; inositol, 50 mg.; vitamin A, 500 I.U.; vitamin D, 100 I.U.; and α-tocopherol acetate, 10 mg. Many of the choline-free diets hitherto employed have been deficient also in vitamin B_{12}, largely because the metabolic significance of this vitamin was not known at the time. The diet herein employed included adequate amounts of this vitamin since we wished to avoid the problems associated with a complex deficiency. In addition, vitamin B_{12} is known to afford some protection against choline deficiency (Schaefer *et al.*, 1949; Hale and Schaefer, 1951) and inasmuch as we wished to test the possibility of enhancement of the deficiency by somatotrophin, it was desirable not to select a diet which gave maximal effects. Eight to fourteen rats caged individually were grouped by weight for each treatment, the average weight ranging from 37–40 gm. The diets and tap water were given *ad libitum* during the period of treatment.

The groups were treated as follows. Group 1 consisted of 14 animals on the basal diet; group 2 received the basal diet supplemented by 0.2% choline; group 3 received the same diet as group 2, but was injected twice daily, subcutaneously, with 1.25 mg. of somatotrophin;[2] group 4 received the same amount of somatotrophin and basal diet; group 5 received basal diet and 2.5 mg. somatotrophin twice daily; group 6 received basal diet and 1 mg. of Hydrocortisone[3] once daily by subcutaneous injection. Somatotrophin was administered as a fresh suspension in physiological saline, the dosage being contained in 0.2 cc. of the suspension. Hydrocortisone was administered in the usual commercial suspending medium, diluted so as to contain 1 mg. in 0.2 cc.

At autopsy the gross renal lesions were listed as +++ (severe), ++ (moderate), + (slight) and 0 (absent). The kidneys and livers were blotted free of blood and weighed while fresh. The former were then placed in Bouin's fixative for histologic examination and verification of pathologic changes, while the latter were ground with anhydrous sodium sulfate and extracted with chloroform for liver lipid determinations.

RESULTS AND DISCUSSION

It was necessary to terminate the experiments involving the deficient diets on the 6th day owing to the poor condition of animals receiving

[2] Somatrofin was obtained from the Frank W. Horner Company, Montreal, Canada.

[3] Hydrocortisone was obtained through the courtesy of Merck and Company, Rahway, New Jersey.

TABLE 1. EFFECT OF SOMATOTROPHIN AND HYDROCORTISONE ON GROWTH RATE, LIVER FAT AND RENAL CHANGES IN RATS ON COMPLETE AND CHOLINE-DEFICIENT DIETS

Data	Group					
	1	2	3	4	5	6
Diet	Basal	Basal +0.2% Choline	Basal +0.2% Choline	Basal	Basal	Basal
Daily Hormone	0	0	2.5 mg. STH	2.5 mg. STH	5 mg. STH	1 mg. Hydrocortisone
Duration—Days	6	7	6	6	6	6
No. Rats						
Started	14	8	9	14	10	10
Survived	13	8	9	13	10 (2 dying)	8
Body Weight—gm.						
Initial	37.7	40.1	37.6	37.3	40.0	38.6
Gain	20.9	24.5†	29.7	20.3	14.8	0.5
Kidneys						
Lesions +++	5	0	0	9	5	0
++	1	0	0	4	2	0
+	4	0	0	0	3	0
0	4	8	9	1	0	10*
Weight—gm.	1.03	0.96	0.94	1.26	1.09	0.79
% Body Weight	1.80	1.34	1.39	2.31	2.10	2.02
Liver Fat						
% Fresh Tissue	9.85	3.20	4.17	9.00	10.64	9.14

* Atypical lesions, see text.
† At six days.

somatotrophin. Those which had not succumbed were afflicted with fine tremors and were obviously extremely ill. The data are summarized in Table 1.

Weight increase. The animals of group 2, receiving the diet containing 0.2% choline, grew normally at a rate of 4.08 gm./day throughout 6 days of treatment. Those given growth hormone in addition, group 3, grew at a rate of 4.95 gm./day. The difference in weight gain between the groups was statistically significant ($P < .005$). Animals on the deficient diet, group 1, grew only at a rate of 3.48 gm./day, an initial rapid gain in the first three or four days being offset by a loss in the severely affected animals in the last two or three days. On this same regimen rats receiving 2.5 and 5 mg. somatotrophin respectively, groups 4 and 5, grew at rates of 3.39 and 2.47 gm./day respectively, the former rate being essentially the same as that observed in group 1, and again showing a terminal weight loss. At the higher dose of growth hormone the weight gain was actually significantly less than at the lower dose. This is probably due to the fact that the greater

metabolic turnover imposed a greater choline requirement. The failure of STH to promote growth in rats on a choline deficient diet is reminiscent of its failure to do so in rats on a pyridoxine deficient diet, thus emphasizing the fact that tissue formation is possible only when adequate materials for synthesis are available. The animals treated with Hydrocortisone did not grow at all during the experiment, but simply maintained their starting weight.

Liver lipid. The accumulation of liver fat, although slight, was observed in all groups on the basal diet. It did not appear to be affected significantly by either growth hormone or Hydrocortisone, since animals so treated exhibited essentially the same percentage of hepatic fat as that encountered in animals on the basal diet. Similarly, the normal liver lipid values observed in rats on the 0.2% choline diet was not affected by simultaneous treatment with growth hormone.

Renal effects. The animals with added choline in the diet were devoid of renal lesions and the kidneys were of normal size, amounting to 1.34% of the body weight. Somatotrophin at a dosage of 2.5 mg./day was without detectable effect on renal structure or size in animals on this diet. Choline deficiency *per se* produced a 71% incidence of renal lesions and a kidney weight which was 1.8% of body weight. On the same diet somatotrophin at a dosage of 2.5 and 5 mg./day increased the incidence of lesions to 93% and 100% respectively, and renal weight to 2.31% and 2.10% of the body weight. However, at the lower dosage the renal lesions were slightly more severe than at the higher dosage, as indicated by the larger size of the kidneys. It would seem that 2.5 mg./day is at or above the maximum potentiating dose.

The kidneys of animals on the deficient diet and receiving 1 mg./day Hydrocortisone acetate grossly appeared entirely normal. While the absolute size was less than that of control animals, on a per cent body weight basis they were larger than control kidneys. This is probably due to the fact that in animals so treated renal growth, while subnormal, continues at a more rapid rate than somatic growth as a whole as is the case in cortisone-treated animals (Hall and Hall, 1952). Microscopically, although renal hemorrhage and the lesions characteristic of choline deficiency were not observed in any of the animals, renal cytoarchitecture was not normal. Glomerular hyalinization of varying degree was present in all of them. It seems doubtful if choline deficiency had anything to do with this, since a high dosage of cortisone is known to produce hyaline changes in the renal glomeruli of rats on normal diets (Selye, 1950). This problem is now under study. The treatment would seem to have suppressed one type of renal lesion and substituted another in its place.

The present data may be briefly epitomized as follows. Weanling male rats maintained on a diet deficient in choline, but adequate in other known nutritional factors, developed a syndrome which included terminal loss in body weight, fatty infiltration of the liver, hemorrhagic necrosis of the renal cortex and increased renal mass. The administration of a purified anterior pituitary growth hormone at 2.5 or 5 mg./day increased both the incidence and severity of the degenerative renal changes and further augmented renal mass in rats on such diets. It did not as obviously affect liver lipid accumulations since the increase was no greater than that in animals on the deficient diet alone. The degree of systemic damage resulting from choline deficiency prevented growth hormone from exerting its somatotrophic effect. Somatotrophin alone, in the presence of an adequate diet displayed no toxicity, produced no renal or hepatic damage and promoted corporal growth. Hydrocortisone at 1 mg./day inhibited body growth, prevented choline deficiency lesions from occurring in the kidney, but did not prevent the accumulation of liver lipid which the deficient diet provoked. A different type of renal alteration, characterized principally by glomerular hyalinization and probably due to the hormone treatment rather than the diet, was observed to occur in choline deficient rats treated with this hormone.

SUMMARY

Anterior pituitary growth hormone at a dosage of 2.5 mg./day augmented the renal damage caused by a choline deficient diet, but did not increase liver lipid accumulation. No greater effect was elicited by doubling the dosage.

Growth hormone while stimulating body growth in animals on an adequate diet was ineffective in preventing the weight loss occasioned by the deficient diet.

Hydrocortisone prevented the typical renal lesions caused by choline deficiency but did not restore a normal cytoarchitecture. It did not prevent the fatty liver observed in animals on the deficient diet.

Acknowledgement

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EFFECTS OF THE MENSTRUAL CYCLE ON THE METABOLISM OF HUMAN ENDOMETRIUM[1]

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THE metabolic mechanisms by which the female sex hormones regulate the structure and function of the tissues of the genital tract have been investigated by three different techniques: (1) the variation in tissue metabolism during normal estrous or menstrual cycles has been measured; (2) the effect on metabolism of hormones injected into castrate animals has been studied; and (3) the effects of steroid hormones on these tissues *in vitro* have been examined. Khayyal and Scott (1935) reported that the metabolism of whole rat uterus varied with the phase of the estrous cycle, and these results have been confirmed (Kerly, 1937). In the human, Raab (1929) found that the oxygen consumption and anaerobic glycolysis of endometrium increased as the menstrual cycle progressed, but Adler (1930) and Dreyfuss (1940) did not confirm these observations. More recently Stuermer and Stein (1952) followed endometrial metabolism is single individuals by means of biopsies. Their data suggest that cyclic variations may exist, although they point out that the results are not statistically significant.

Since these investigations of human endometrium were performed with different incubation media and techniques it is not surprising that the values obtained for the metabolic properties measured varied greatly from one report to the next. It is clear, however, that there is an inherent wide variability in metabolism from one specimen of endometrium to another.

Among others, Roberts and Szego (1953) have studied the effects of injected hormones on the metabolism of whole rat uterus. They found that estrogens stimulate both oxygen consumption and anaerobic glycolysis in this organ. The addition of pure estrogenic hormones to whole rat uterus *in vitro* apparently has no effect (Kerly, 1940, Sweeney, 1944) but it has

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been demonstrated (Hagerman and Villee, 1953) that estradiol can influence directly the metabolism of human endometrium *in vitro*.

In the investigations mentioned above in which stimulatory effects of estrogens were observed, there was an increase in oxygen consumption by the tissue, usually associated with an increased rate of substrate utilization or oxidation. The metabolic effects of progesterone have not been as clearly delineated, although in the whole animal the actions of the hormones are complementary. The data presented in this paper show that cyclic variations in the metabolism of human endometrium exist, and that the metabolic effects of estrogen *in vivo* and *in vitro* are identical.

METHODS

The tissue used in these experiments was obtained from uteri removed for reasons other than clinically evident endometrial pathology, and was judged to be histologically normal.[3] The specimens were dated with respect to the last menstrual period from the clinical record and from histologic criteria, and were classified in an arbitrary 28-day cycle. The methods of incubation and chemical analysis have been previously described

TABLE 1. EFFECTS OF THE MENSTRUAL CYCLE ON THE METABOLISM OF HUMAN ENDOMETRIUM *in vitro*

Period	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	P[a]
Days of Cycle	1–5	6–10	11–13	14–16	17	18	19	20–21	22–24	25–28	Menopausal	
Q_{O2}	1.97	3.49*	2.37	1.98	1.91	2.68	2.84	2.21	3.46	2.48	1.28	<0.01
Change in Tissue Glycogen[b]	−1.0	+0.2	+0.1	+0.2	+0.3	−5.2	−5.6	−2.1	−3.4	−4.0	0	<0.01
Glucose Utilization	17.7	17.7	9.4	9.4	14.7	15.9	9.2	9.3	3.8	5.7	30.5	<0.05
Pyruvate Utilization	19.9	24.6	8.7	43.0	13.2	19.3	23.5	19.0	17.3	21.6	24.0	—
Lactate Production	48.8	26.5*	23.2	27.0	36.2	25.8	26.8	36.4	20.4	22.8	30.6	<0.01
Glucose Oxidized	0.11	0.29	0.13	0.25	0.17	0.16	0.18	—	0.20	—	—	—
Pyruvate Oxidized	0.5	3.3†	2.1	2.0	0.9	2.8	3.1	1.3	2.2	—	—	<0.01
% CO_2 from Glucose	1.1	1.6	1.8	1.4	1.7	4.4	2.3	—	0.9	—	—	<0.01
% CO_2 from Pyruvate	2.8	19.4	16.5	7.8	6.3	22.3	16.9	13.3	5.8	—	—	—

Values are given as means in micromoles/gm. wet wt./hr., except Q_{O2}'s which are microliters/mgm. dry wt./hr., and fraction of respired carbon dioxide derived from the radioactive substrate which are percentages.

[a] Probability that the means of periods I to XI are drawn from the same population.

[b] Negative numbers signify utilization of glycogen by the tissue.

* $P < 0.05$, † $P < 0.01$ } that period II is drawn from the same population as period I.

(Hagerman and Villee, 1953, Villee, White and Hastings, 1952). The substrate used was a mixture of potassium pyruvate (10 millimoles/liter) and glucose (5.5 millimoles/liter). Samples from each specimen were incubated in duplicate in the absence and in the presence of estradiol[4] (4×10^{-6} moles/liter), and in most of the experiments radioactive pyruvate and glucose[5] were added to alternate vessels so that the eventual fate of these substrates could be traced.

The data for the two groups of determinations (with and without added estradiol)

[3] We are indebted to the Staff members of the Free Hospital for Women and the Boston Lying-in Hospital for the tissue specimens used, and to Drs. A. T. Hertig, D. G. McKay and H. Mansell for the results of the histologic examinations.

[4] Crystalline estradiol-17β was generously provided by the Schering Corp.

[5] Uniformly labeled C^{14}-glucose was obtained from the Atomic Energy Commission, Oak Ridge, Tenn. Potassium pyruvate-2-C^{14} was prepared by Dr. M. L. Karnovsky, Biophysical Laboratory, Harvard Medical School.

TABLE 2. VARIATION IN THE *in vitro* EFFECTS OF ESTRADIOL ON HUMAN ENDOMETRIUM WITH THE MENSTRUAL CYCLE

Period	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	P[a]
Q_{O_2}	2.30	3.33	3.10	2.45	2.16	2.96	2.62	2.94	3.70	2.35	1.46	<0.05
Change in Tissue Glycogen[b]	−0.4	+0.1	−1.0	−0.3	−4.3	−6.3	−4.9	−2.0	−1.8	−4.6	0	—
Glucose Utilization	19.7	12.2	6.8	24.4	11.3	14.6	4.6	12.5	8.4	4.6	18.4	—
Pyruvate Utilization	15.4	31.7	9.6	15.8	12.3	16.6	20.6	23.2	30.1	18.1	13.2	—
Lactate Production	56.0	24.0	21.8	22.4	38.5	25.9	21.3	45.9	16.4	17.2	29.6	—
Glucose Oxidized	0.17	0.45	0.30	0.20	0.16	0.28	0.16	—	0.29	—	—	—
Pyruvate Oxidized	0.9	2.9	2.6	2.0	1.3	2.3	2.4	1.4	1.7	—	—	—
% CO_2 from Glucose	1.4	2.3	4.0	1.6	1.5	5.2	2.2	—	1.3	—	—	<0.05
% CO_2 from Pyruvate	3.0	22.2	15.5	7.4	8.5	21.7	15.5	18.3	27.2	—	—	—

Values are given in the same units as in Table 1. Each value in this table, obtained in the presence of estradiol, corresponds to the same value in Table 1 determined in the absence of estradiol.

[a] Probability that the mean of the values for all periods of this table is drawn from the same population as the corresponding value in Table 1; i.e., that there is no *in vitro* estradiol effect.

[b] Negative numbers signify utilization of glycogen by the tissue.

were divided into eleven periods according to phase of the menstrual cycle (see Table 1 for the days of the cycle represented in each period). The data were examined statistically by an analysis of variance (Gore, 1952). Each value in Tables 1 and 2 represents the mean of four determinations, while each of the points in Figure 1 is the mean of 2 to 8 determinations.

RESULTS AND DISCUSSION

The variation in endometrial solids and glycogen content is illustrated in Figure 1. The variations are statistically significant at the 1% confidence level. The observed fall in solids content during the early part of the cycle, followed by a later increase is similar to the estrogen induced imbibition of water followed by a phase of growth in the rat uterus, and the low solids content observed late in the cycle is in agreement with the histologic

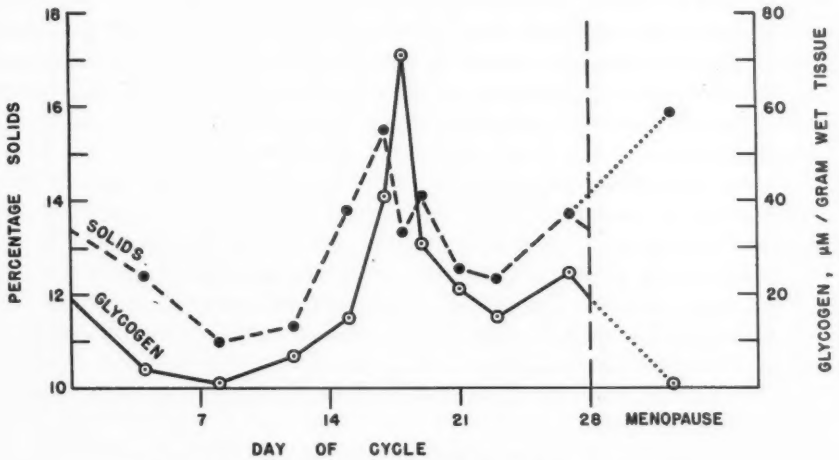

FIG. 1. Variation in solids and glycogen content of human endometrium with the menstrual cycle.

"edema" seen in human secretory endometrium. Stuermer and Stein (1951) found, in a smaller series of determinations, no such variation in water content of human endometrium.

The glycogen content of these specimens paralleled the total solids content. These chemical determinations are similar to previous analyses (cf. Reynolds, 1949) and to the results of histochemical examinations, but the sharp decrease observed during the last quarter of the cycle has apparently not been previously described.

In the menopausal specimens the glycogen content was very low, and the solids content was quite high, in keeping with the afunctional state of the tissue at this time.

The data recorded in Table 1 show how the metabolism of carbohydrate by endometrium varies with the menstrual cycle. All the metabolic functions measured except the rates of pyruvate utilization and glucose oxidation, and the fraction of respired carbon dioxide derived from pyruvate showed statistically significant variations. The rate of oxygen consumption showed two peaks, one in the proliferative phase (period II) and another in the secretory phase (period IX), in agreement with the findings of Stuermer and Stein (1952).

The rate at which glycogen was utilized was proportional to the glycogen content of the tissue at the beginning of the experiment (cf. Fig. 1), for early endometrium synthesized small amounts of glycogen but later in the cycle there was a marked net utilization. However, even in the experiments with proliferative endometrium where there was a net synthesis of glycogen as measured chemically, the amounts of radioactive substrate incorporated into glycogen were too small to permit accurate measurement. This indicates that both the synthetic and degradative reactions were proceeding quite slowly. In secretory endometrium degradation went on so rapidly that any synthesis which may have occurred was completely obscured.

The rate of glucose utilization decreased somewhat during the late proliferative phase, rose slightly about the eighteenth day of the cycle and reached a minimum in the latter part of the secretory phase. The lactic acid produced in these experiments was derived partially from the stored glycogen and partially from the glucose and pyruvate of the medium. Its rate of production was, therefore, the resultant of a number of complex factors which cannot be resolved. The rate fell early in the cycle (period II) and remained low with slight intermediate fluctuations until the beginning of the next cycle.

The amount of pyruvate oxidized and the fraction of respiratory carbon dioxide derived from glucose showed statistically significant variations with the cycle, and the companion figures for glucose oxidation and fraction of

respiratory carbon dioxide derived from pyruvate were parallel. All these measures of substrate oxidation rose to a maximum in the proliferative phase (periods II and III), fell during midcycle, and rose again in the latter half of the cycle.

The overall picture presented by these data is one of a biphasic stimulation in metabolism during the cycle with the peaks occurring around the sixth to eighth and the seventeenth to twentieth days. The uncontrollable genetic and environmental differences inherent in the experimental material make the data highly variable and it might be argued that although the data show statistically significant differences, they may not be related to physiologic reality. However, the parallelism between these metabolic changes and the amount of estrogen present in the circulating blood stream is probably more than coincidence. Only the variations in glycogen metabolism do not fit this pattern but it seems reasonable to suppose that an estrogen stimulated synthesis of glycogen early in the cycle is modified by the action of progesterone later. Since it has not yet been possible to demonstrate *in vitro* effects of progesterone on endometrial metabolism (Hagerman and Villee, 1953), this explanation remains speculation.

The data for period XI (menopausal endometrium) indicated that this tissue was metabolizing at a slow rate. The relatively high rate of glucose utilization and low oxygen consumption suggest that here the metabolism is primarily glycolytic.

A direct comparison of periods I and II is also indicated in Table I. Here the *in vivo* effects of estrogen uncomplicated by the presence of progesterone can be seen. Even in the small number of samples represented in the two periods there was a significant increase in oxygen consumption and pyruvate oxidation with a decrease in lactate production. These effects are similar to those described by Roberts and Szego (1953) for whole rat uterus from previously estrogen injected animals. Likewise, they are similar to the effects of estradiol on human endometrium *in vitro* (Hagerman and Villee, 1953) and on human placenta slices and homogenates.[6]

The data of Table 2 show how the *in vitro* effects of estradiol on endometrium are altered by the previous history of the tissue. The probabilities in the last column of Table 2 refer to the *average* effect of estradiol throughout the entire menstrual cycle and are similar to those we have already reported. However, the fact that oxygen consumption and glucose oxidation are increased on the average by estradiol *in vitro*, does not give the complete picture. There exists in addition a reciprocal relationship between the rate of endogenous metabolism and the degree to which it can be stimulated *in vitro*. In period I, for example, estradiol *in vitro* stimulates oxygen

[6] Unpublished observations.

consumption 17%, while in period II the hormone is ineffective. The amount of glucose oxidized is stimulated to the same extent (54%) in both these periods, while pyruvate oxidation is capable of an 80% stimulation in period I and none in period II. It would appear that, whatever the source of the estrogen, endogenous or exogenous, endometrial metabolism can be hormonally stimulated to approximately the same extent. Moreover, the nearer the metabolic process measured lies to the Krebs tricarboxylic acid cycle, the greater is the magnitude of the observed estrogen-induced stimulation. The Krebs cycle is the final stage of carbohydrate metabolism to carbon dioxide and water, and the apparent site of action of estradiol *in vitro* (Hagerman and Villee, 1953).

The biochemical mechanisms definable in simple systems such as tissue slices, homogenates, etc., must eventually be related in some fashion to the mechanisms active in the whole body. The concept that hormonal control of the morphology and physiology of endometrium is mediated via alterations in the fundamental metabolism of the cell is well established by the work of Roberts and Szego (1953) and many others. The present data demonstrate that the effect of endogenous estrogen *in vivo* (as measured by cyclic metabolic variations with the menstrual cycle) is similar in man to the effects of endogenous and exogenous estrogen in rodents, and that these effects are also similar to those observed when estradiol is added to human endometrium in an *in vitro* system.

SUMMARY

Human endometrium, incubated *in vitro* shows cyclic variations with the menstrual cycle in oxygen consumption, glycogen utilization, lactate production, amount of pyruvate oxidized and fraction of respiratory carbon dioxide derived from glucose. Determinations of total solids content and glycogen content of human endometrium show that these also vary with the menstrual cycle. The metabolic changes can be correlated with variations in the amount of estrogen available to the tissue *in vivo* prior to the experiment. The *in vivo* effects of estrogen deduced from these variations are in the same direction and of the same magnitude as those induced by estradiol added to endometrium incubated *in vitro*. It would appear, therefore, that the mechanism of action of the hormone is identical in both these experimental situations.

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QUALITATIVE CHANGES IN URINARY GONADOTROPHINS IN HUMAN PREGNANCY DURING THE PERIOD OF RAPID INCREASE IN HORMONE TITER[1]

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THE gonadotrophic action of the urine of pregnant women was first shown in normal immature mice and rats in which it caused enlargement of follicles and corpus luteum formation. Later, when pregnancy urine was concentrated and detoxified, it was possible to inject it into hypophysectomized immature female rats. Its only effect on the ovary of such animals was on the interstitial cells; no follicular growth or corpus luteum formation occurred. As many as 8000 units (judged by the ability to stimulate corpus luteum formation in immature normal rats) have been injected by Evans and Simpson into hypophysectomized female rats with no other gonadotrophic action than interstitial tissue stimulation. Comparable results were obtained by Claesson *et al.*, 1948, with 6000 I.U. of highly purified HCG injected over a 4 day period and as many as 24,000 I.U. over a 16-day period. The complete gonadotrophic response to pregnancy urine observed in the normal immature animal must, then, be indirect through the pituitary. This mechanism might involve either stimulation of the pituitary, or synergic action with a pituitary hormone present in amounts inadequate to be effective alone. Synergism is known to occur between pituitary follicle stimulating hormone (FSH) and the hormone present in the urine of pregnant women (HCG). In fact, about 1/5 the dose of either substance which is effective alone can be detected by the combination with adequate amounts of the other (Evans and Simpson 1950). As synergism is shown in hypophysectomized as well as in normal rats, the conclusion must be drawn that the difference between the response of the normal and that of the hypophysectomized animal to pregnancy urine is primarily due to the contribution of FSH from the pituitary, occurring in the normal.

The evidence is ordinarily considered adequate that the origin of the gonadotrophic hormone in blood and urine of pregnant women is from the

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chorion. It is assumed that the low titer of gonadotrophin present in urine of normal women (non-pregnant) is of pituitary origin. In this instance, hormone is present in such small amounts that it has proved difficult to free urinary concentrates sufficiently from toxic substances to be able to inject them in adequate amounts in hypophysectomized animals. The few studies that have been made (e.g. Bahn *et al.*, 1953) indicate that normal urine contains pituitary-like follicle stimulating and interstitial cell stimulating substances.

The gonadotrophic hormone of pregnancy is described as appearing in the urine a few days prior to the expected menstrual period, within a few days after implantation, or in the third week of the cycle. The time has been placed at 5 days before the expected menses by Smith, Albert and Randall, 1951. It has been placed between 10 and 13 days after fertilization by Browne and Venning, 1936, and by Tenney and Parker, 1937. The time of the increase in hormone output has been described by Smith and Smith, 1936, on the 23rd to 24th day of the cycle; by Levin, 1941, on the 23rd day of the cycle; and by the Pedersen-Bjergaards, 1948, on the 20th to 21st day of the cycle. After a somewhat slow start, the concentration rises abruptly to a maximum, which is usually described as occurring between 50 and 70 days after the last menstrual period (Browne and Venning, 1936; Evans, Kohls and Wonder, 1937; Smith, Albert and Randall, 1951).

The objectives of the present study were first to determine accurately the time of the early rise in gonadotrophic titer, as measured from the onset of the last menses, or preferably from the time of ovulation. Using these data, the qualitative changes in types of gonadotrophic hormone excreted could then be examined at the time of the abrupt increase in concentration.

Assays of the urinary titers were conducted at frequent intervals beginning shortly after ovulation on three of the 16 normal pregnant women studied (Fig. 1, patients C.B., P.M., E.W.) in order to determine the time and extent of the change in gonadotrophic excretion. The titer was measured in terms of mouse uterine units for 24 hour excretion periods.[2] The

[2] The assays were carried out in 21-day old females of the Webster Strain of Swiss white mice. Three or preferably six mice were used for every dose level. Each mouse received the total dose over 3 days, given as 0.5 cc. injected subcutaneously once daily, followed by autopsy 72 hours after the first injection. An increase of 100% in the weight of the drained uterus over that of the uninjected control constituted a positive response. The uteri of controls were described as small or small medium. They ranged in weight from 7 to 29 mg. and averaged 15 mg. (90 mice). An average uterine weight of 40 mg. was considered a minimal response. Such uteri were described at autopsy as large, or estrous. One I.U. either of the International standard or of a purified preparation of HCG (prepared by Parke, Davis and Co. and kindly furnished to us by Dr. D. A. McGinty) was equivalent to 1 M.U.U. as determined by this assay.

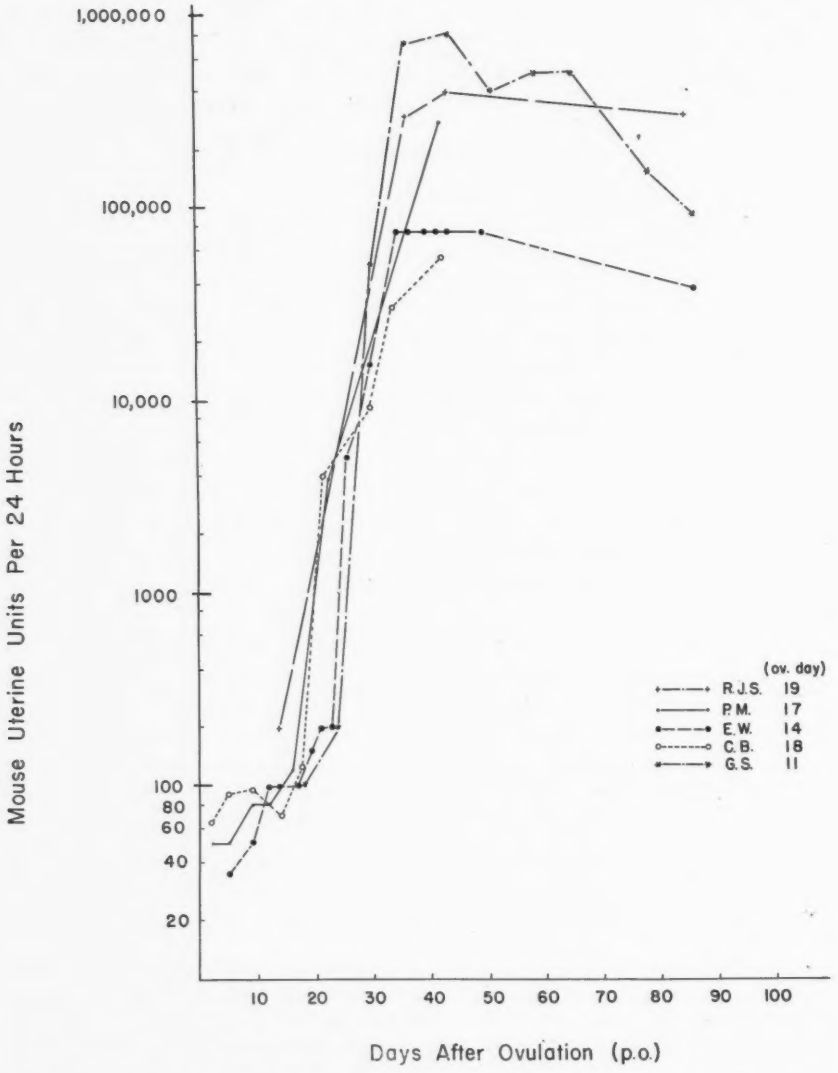


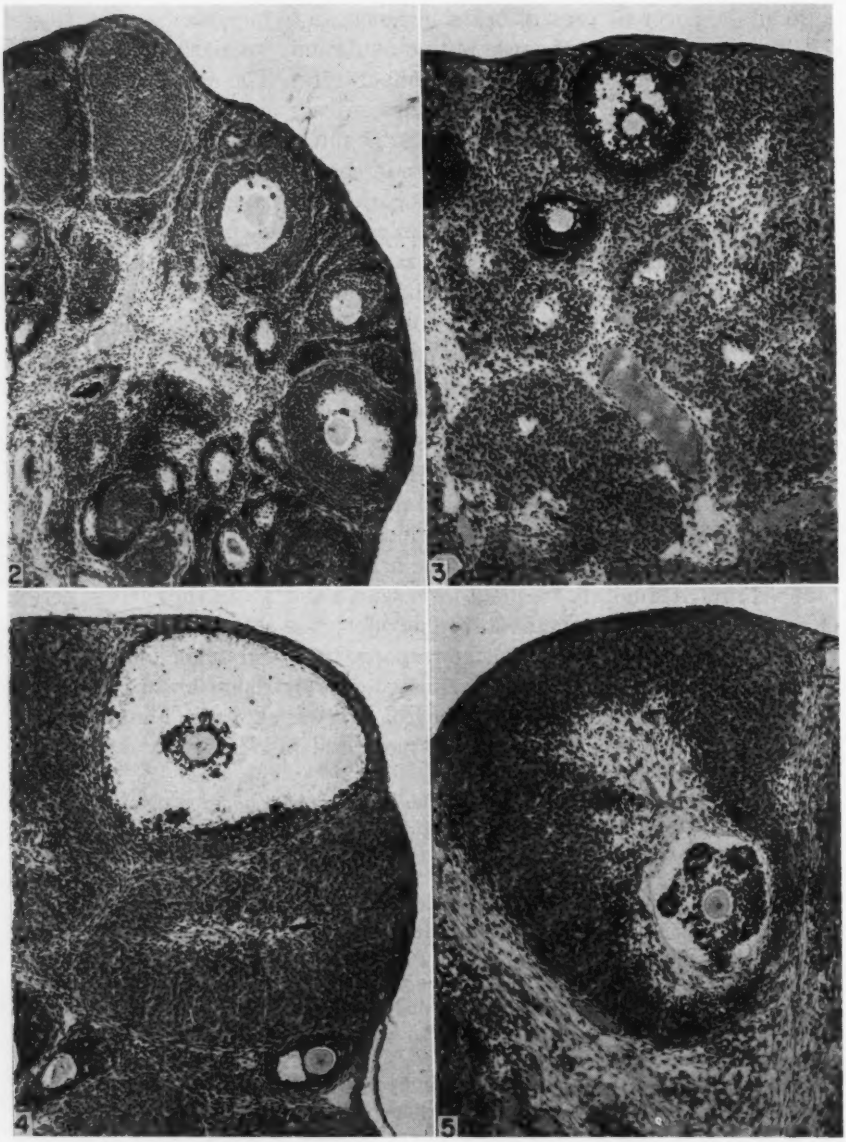
FIG. 1. Graph showing the relation between the urinary gonadotrophic hormone titer, in mouse uterine units (logarithmic scale) and the time of pregnancy calculated from the day of ovulation in five selected cases.

amounts of hormone present in the urine began to increase between 10 and 11 days after the time of presumptive ovulation, (ovulation time was determined by the rise in basal body temperature). The actual intervals before the titer began to rise in these three women were respectively 10, 10 and 11 days post ovulation (p.o.) or 28, 27 and 25 days from the beginning of the last menses (p.m.). The increase in concentration of hormone in the urine was rapid. Plotted on a semi-logarithmic scale, the curve approximated a linear relationship between time and the log of the concentration. The titer reached 40,000 to 400,000 M.U.U. between 35 and 40 days p.o. as can be seen in Figure 1. Included in Figure 1 are two additional curves (G.S. and R.J.S.) showing gonadotrophin titers early in pregnancy, though not prior to the first missed menstrual period.

In order to analyze the type of gonadotrophic hormone excreted, urines from 16 women in various periods of pregnancy, including the 5 represented in Figure 1, were tested in hypophysectomized immature female rats.[3] In each assay the equivalent of 1/5 of the 24-hour urine was injected into each of three hypophysectomized rats.[4] In one patient, E.W., in which urines had already been assayed in mice at thrice weekly intervals from the 5th day p.o. (day 19 of cycle), the urines were assayed in hypophysectomized rats at similarly frequent intervals (Table 1). In the earliest period of pregnancy in which a gonadotrophic effect was shown, that is, from the 12th day p.o., or 26 days p.m., the response observed in the ovaries of the hypophysectomized rats was, as expected, merely repair and hypertrophy of interstitial tissue (Fig. 3). The control ovary is shown in Figure 2. However, when the titer of hormone, as measured by the intact mouse, had

[3] The entire 24 hour urine specimen, which had been kept under refrigeration during the collection period, was filtered and brought to pH 4.6 by the addition of 5*N* HCl. It was added, with stirring, to four volumes of cold 95% ethanol. The precipitate was allowed to settle overnight in the cold (2 to 5° C.), after which the supernatant was decanted or siphoned off. The precipitate was then washed 3 times with cold 95% alcohol, with centrifugation between, and once with cold 100% alcohol (these procedures being carried out at room temperature but with chilled reagents and a chilled centrifuge). After washing twice with absolute ether, the precipitate was dried rapidly. It was then dissolved in a small volume of water and dialyzed overnight in running tap water at 2–5° C. and 1–2 hours in distilled water. After centrifugation the supernatant was dried by lyophilization.

[4] The qualitative assay was carried out in hypophysectomized 26–28 day old female rats of the Long-Evans strain, one week postoperatively. Three rats were used for each test. A weighed amount of the dried powder was dissolved in distilled water so that each total dose of 1/5 of a day's output was contained in 4 cc. This was injected as 1 cc. subcutaneously once daily for 4 days, with autopsy at 96 hours. The dissected ovaries were weighed, fixed in Bouin's fluid, embedded in paraffin, sectioned serially at 8μ and stained with hematoxylin and eosin. Weights of drained uteri were also recorded as supplementany evidence of ovarian activity.



Ovaries of hypophysectomized, 26–28 day old female rats, 10–12 days postoperative. H and E stain, ×125.

TABLE 1. RESPONSE OF THE OVARIES OF IMMATURE HYPOPHYSECTOMIZED RATS TO CONCENTRATES OF HUMAN PREGNANCY URINE

Day,* p.o.	M.U.U., 24 hr.	Ovaries†			Wt., mg.	Uterus Wt., mg.	Thyroid (3 rats)		
		Follicles	Corpora lutea	Interstitial tissue					
5	35	No F devel.	none	deficient	8	27			
9	50	No F devel.	none	deficient	10	30			
12	100	No F devel.	none	repair	22	31			
14	100	No F devel.	none	hypertrophy	20	43	−	+	+
16	100	No F devel.	none	hypertrophy	24	36	−	−	−
19	150	1F	lut. F	hypertrophy	29	53	−	+	+
21	200	m, 1F	lut. F	hypertrophy	25	43	−	−	+
23	200	sF		hypertrophy	18	38	−	−	+
26	5,000	1F	CL	hypertrophy	44	81	−	+	+
28	7,500	1F	CL	hypertrophy	73	108	+	+	+
30	15,000	1F, cysts	CL	hypertrophy	88	164	−	+	+
33	15,000	1F, Bl. Pt.	CL	repair	57	119	−	+	2+
35	75,000	1F, cysts	CL	repair	123	154	−	+	+
37	75,000	1F, cysts	CL	repair	109	119	+	2+	2+
40	75,000	1F	CL	repair	58	117	+	+	2+
42	75,000	1F	CL	repair	129	142	2+	2+	2+
44	75,000	1F	CL	repair	76	120	+	2+	2+
50	75,000	1F, cysts	CL	repair	122	134	2+	2+	2+
121	20,000	sF	none	hypertrophy	22	34			
176	40,000	s, mF	none	hypertrophy	24	48	−	−	−
203	20,000	s, m, 1F	lut. F	hypertrophy	60	109	−	−	−
238	40,000	s, m, 1F	lut. F	hypertrophy	28	50	+	+	+
	Control	sF few	none	deficient	10	22	−	−	−

* Day post-ovulation. Patient E.W., presumptive ovulation cycle day 14.

† Conditions of the test are given in footnote 4. Organ weights listed are averages from 3 rats. The abbreviations s, m, 1F indicate small, medium and large follicles; CL indicates corpora lutea.

EXPLANATION OF FIGURES 2–5

FIG. 2. Untreated control. No follicles developed beyond the stage of beginning antrum formation; interstitial tissue deficient. Weight of ovaries 10 mg.

FIG. 3. Injected for 4 days with a total dose of 1/5 daily urine output from day 16 p.o. or 30 p.m. No follicular development; interstitial tissue hypertrophied. Weight of ovaries 29 mg.

FIG. 4. Injected for 4 days with a total dose of 1/5 daily urine output from day 28 p.o. or 42 p.m. Large follicles, corpora lutea and hypertrophied interstitial tissue. Weight of ovaries 73 mg.

FIG. 5. Injected for 4 days with a total dose of 1/5 daily urine output from day 35 p.o. or 49 p.m. Cumulus oophorus persisting in a luteinized follicle. Interstitial tissue repaired and edematous. Weight of ovaries 146 mg.

reached 5000 M.U.U., that is, 26 days p.o. or after the 40th day of pregnancy, then the urinary concentrates regularly induced follicular growth[5] and corpus luteum formation, as well as interstitial cell repair (Figs. 4 and 5). The amount of follicle stimulating hormone in the urinary concentrates increased, as judged by the number of enlarged follicles, and size of the ovaries and uterus, until a maximum was reached 35 days p.o. (49 days p.m.). This maximum was reached at the same period at which the mouse uterine unitage had reached the highest values. Thereafter, follicle stimulation was observed in almost all tests made on this patient, to 238 days p.o. (252 days p.m.). The concentration of the follicle stimulating principle present decreased in later pregnancy, judged by the ovarian weights and follicular size. No attempt was made to quantitate the unitage in hypophysectomized rats.

In 5 other patients it was shown by the hypophysectomized test animal that an interstitial tissue repairing hormone was present in urine by 15 to 18 days p.o. (see Table 2). A follicle stimulating substance was observed in the urine of 12 of 14 women in which adequate tests were made in hypophysectomized rats.[6] These tests covered various periods from 35 to 252 days of pregnancy. Table 2 shows the time of pregnancy when follicle stimulation was first observed. It can be seen by comparison with Table 1 that follicles were stimulated regularly in the hypophysectomized test animals when the titer of the urine injected had reached 5000 to 10,000 M.U.U., and that follicular development was most marked when the quantitative titer was highest, i.e. between 40 and 70 days of pregnancy.

The detection of a follicle stimulating principle in pregnancy urine was unexpected, as no well authenticated evidence of such a hormone had been presented previously. Attention is directed to the fact that the procedure used for concentrating the urine made it possible to inject a larger fraction of a day's output in hypophysectomized rats, without toxic effects, than had previously been feasible.

A thyroid stimulating substance was also present in the pregnancy urines tested (Figs. 6 and 7). In the most thoroughly tested patient (E.W.) the thyrotrophic substance appeared in the urine as early as the follicle stim-

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[5] Follicle growth was observed earlier—by the 19th day p.o. (33 p.m.) when the titer was 150–200 M.U.U. per 24 hours—but was not constantly present until the titer was much higher.

[6] Regarding the absence of follicle stimulating hormone from the urine in 2 of the 15 women studied, it should be noted, that in one the urine was tested once only, late in pregnancy (day 211 p.m.). In the other the only test was on urine collected day 56 p.m., and abortion occurred 10 days later. In two other patients, of the total 16 of this study, no adequate examination was made of the urine in hypophysectomized rats after the gonadotrophic titer had risen to a high level.

TABLE 2. TIME OF APPEARANCE IN PREGNANCY URINE OF INTERSTITIAL CELL STIMULATING AND OF FOLLICLE STIMULATING EFFECTS
1/5 Daily Urine per Hypophysectomized Rat

Patient	Repair or hypertrophy of interstitial cells		Follicle stimulation and interstitial repair	
	days p.o.	days p.m.	days p.o.	days p.m.
EW	>9<12*	>23<26	>14<19	>28<33
ER		<34		<34
RJS	<15	<34	<37	<56
PM	>12<16	>29<33	— —†	— —
CB	>14<18	>32<36	— —†	— —
GS	<18	<29	>23<31	>34<42
LS	‡	<38		<38
BM	<22	<43	>22<45	>43<66
OB		<56		absent 56 (aborted 68)
KS		<68		<68
VK	<55	<69	<55	<69
JO		<73		<73
NB		<91		<91
CP	<84	<97	<84	<97
VS		<127		<127
MM		<211		absent 211

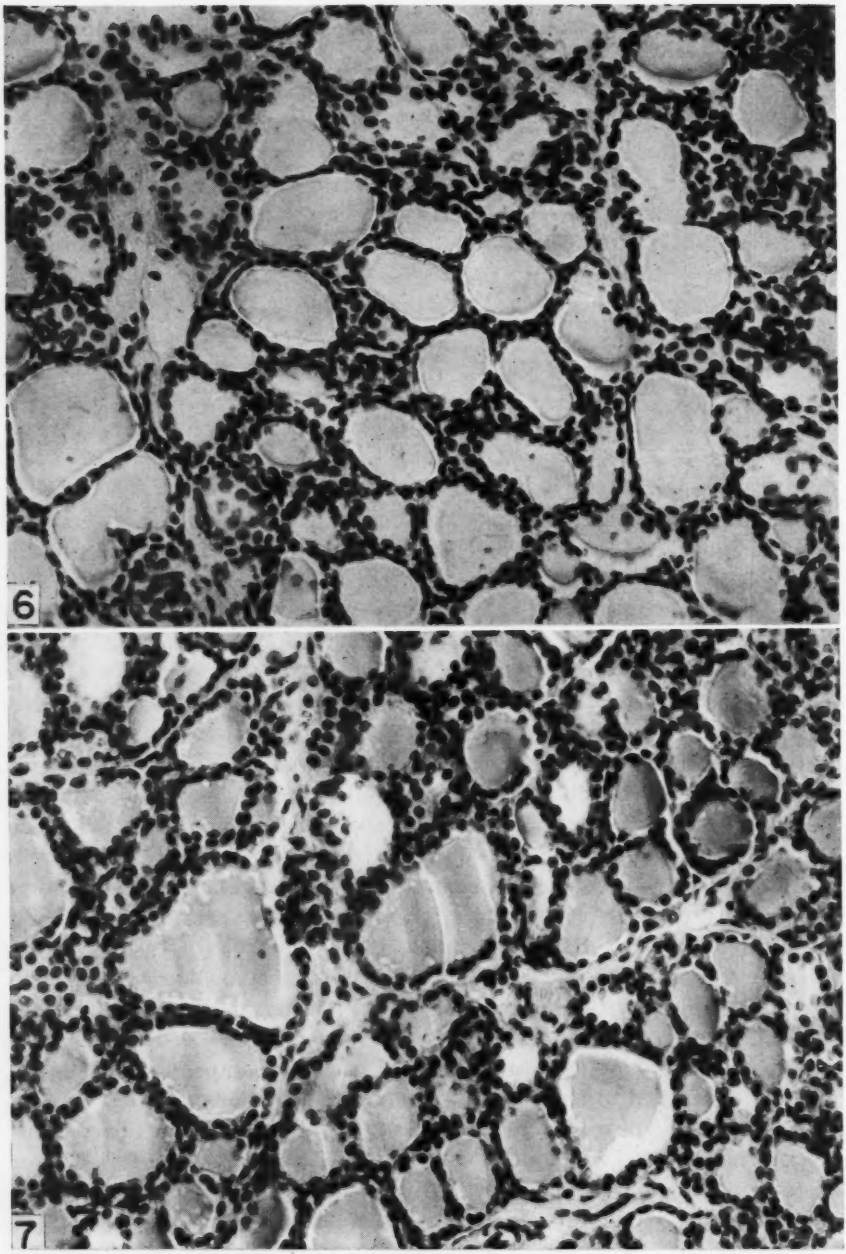
* When two figures are given in a column they indicate that the hormone appeared after the first and before the last time indicated. When one figure is given it shows that tests were infrequent and that the hormone appeared before that date.

† These are cases which were inadequately tested in hypophysectomized rats after the great increase in gonadotrophic titer had occurred.

‡ Blanks are left where time of ovulation was not known.

ulating hormone and was detected into late pregnancy (Table 1). The response of the thyroids of the hypophysectomized rats was seen most consistently when the ovaries showed the greatest follicular stimulation. Thyroid stimulation was observed on injection of urine concentrates from 12 of the 14 patients so examined. No decisive reaction of the adrenal cortex of the test animals was detected either from adrenal weights or adrenal morphology in Sudan black stained frozen sections. The tibia test showed no evidence of growth hormone in any urine concentrate tested in hypophysectomized rats.

In an attempt to understand why purified preparations of chorionic gonadotrophin made from pregnancy urine had not previously been found to induce follicular growth in hypophysectomized rats, a reexamination was made under the identical conditions used for assays in these studies. A highly purified preparation of chorionic gonadotrophin, containing 2500 I.U. per mg. prepared by Parke, Davis and Company was injected into hypophysectomized immature female rats. A total of 20,000 I.U. were injected in daily subcutaneous doses over a 4-day period. Under these conditions follicular stimulation did occur. In addition to hypertrophied interstitial tissue, the ovaries contained many growing follicles, medium to large in size, most of them showing thecal luteinization. Some solid luteinized



Thyroids of hypophysectomized 26–28 day old female rats, 10–12 days postoperative. H and E stain, ×360.

structures, the size of mature corpora lutea, but containing enclosed ova, were also present. The ovaries were increased in weight from 8 mg. in controls to 129 mg. The uteri were enlarged and had been increased in weight from 23 to 162 mg. At half this dose, 10,000 I.U., the ovaries contained fewer large follicles and the ovarian weights were not so large, 86 mg. The average uterine weight was 143 mg. A low level of thyroid stimulation resulted from injection of both dose levels but no adrenal stimulation was observed.

The follicular development shown by hypophysectomized rats receiving concentrates of pregnancy urine may have resulted from contaminating pituitary hormone, which, though present in low concentrations, became evident when such large doses were injected. Usually the human pituitary, like the pituitary of other mammals, is supposed to be low in gonadotrophic hormone during pregnancy. (Phillipp in 1930 first tested such pituitaries and was unable to detect gonadotrophic hormone. Bruner, 1951, is in agreement with this finding.) It is quite possible, however, that well-preserved pituitaries from normal pregnant women have not been adequately tested.

The appearance in the urine of a thyrotrophic substance in addition to the follicle stimulating substance would be considered by some to point to the production, secretion and excretion of these pituitary trophic hormones. However, it is also possible that the placenta may have produced the follicle stimulating and thyrotrophic hormones in addition to the chorionic gonadotrophin. The content of these substances in the placenta has not been adequately investigated. It is possible to report the assay in hypophysectomized rats of 3 early placentas.[7] One was removed intact on the 61st cycle day, a period of peak hormone production judged from urine. The ovaries of the test animals showed follicular development, corpus luteum formation and interstitial tissue repair. This is, however, not conclusive evidence that the follicle stimulating substance present in the placenta was produced there. It is possible that it was derived from circulating hormone of pituitary origin. Neither thyroid stimulating nor adrenal stimulating substances were found.

The other two placentas tested were from cycle day 68, one a spontaneous and the other a therapeutic abortion. In both, the embryo was removed

EXPLANATION OF FIGURES 6–7

FIG. 6. Untreated control. Note low cuboidal or squamous type of lining epithelium, and compact colloid.

FIG. 7. Injected for 4 days with a total dose of 1/5 daily urine output from day 42 p.o. or 56 p.m. of pregnancy. Note increased height of epithelium, beginning vacuolation of colloid.

and the placental tissue dissected free of all residual tissue and adherent blood, which was then assayed separately. No follicle stimulating or thyroid stimulating effect was obtained from these placentas, but there was marked interstitial tissue hypertrophy. The same results were obtained from assay of the residues. Under the conditions of these experiments, no morphological evidence was obtained of the presence of thyrotrophic hormone (from histology of the thyroid) or of adrenotrophic hormone from the adrenal repair test in hypophysectomized rats.

No follicle stimulating or thyroid stimulating substance was detected by the hypophysectomized rat test in the blood sera of 5 women, 30 to 230 days of pregnancy when the untreated sera were injected (daily, subcutaneously over a 4-day period) in a total volume of 1 to 4 cc.

The presence of follicular growth as well as interstitial repair in the ovary of the hypophysectomized rat treated with pregnancy urine might be subject to the same interpretation as the tubular differentiation and Leydig cell stimulation which occur simultaneously in the testes of hypophysectomized rats injected with chorionic gonadotrophin (Smith and Leonard, 1934; Evans, Pencharz and Simpson, 1934). It could be assumed that chorionic gonadotrophin is a complete hormone having both gametokinetic and interstitial cell stimulating properties. (The more commonly accepted explanation of the gametokinetic action of HCG in the male rat is that it is secondary to androgen produced by the stimulated Leydig

[7] The tissue obtained from therapeutic abortion was fixed in cold acetone immediately after removal at hysterotomy; that from the spontaneous abortion was fixed in acetone as soon as received. The acetone was removed from the precipitate by dialysis and the product was dried by lyophilization. The dry weights of placental tissue and residues are shown in tabular form. An aqueous extract was made of the ground product and a total dose, representing one fourth of a placenta (or an equivalent weight of residue) was injected into each of three hypophysectomized rats. This was given in single daily injections over a four day period. Ovarian and uterine weights are also shown in the table. The lack of follicle stimulation from the two 68 day placentas may have been due to the lower dose injected.

Placenta	Cycle day	Dry weight of product	Weight extracted, injected per rat	Organ weights	
				Ovaries	Uterus
		gm.	gm.	mg.	mg.
Therapeutic Abortion	61	0.47 placenta & residue	0.12	63	81
Therapeutic Abortion	68	0.23 placenta 10.90 residue	0.06 0.06	24 34	33 40
Spontaneous Abortion	68	0.22 placenta 2.56 residue	0.05 0.05	16 31	25 35
Control	—	0.0	0.0	13	23

tissue.) If one does accept the idea that HCG is a complete gonadotrophic hormone, effective in both males and females, then one would necessarily assume that the follicle stimulating properties of the hormone are extremely low. Widely differing proportions of follicle stimulating and interstitial cell stimulating properties have been shown in gonadotrophic preparations from different sources (Simpson, 1944). The hormone most similar to HCG would be pituitary ICSH, of which more than 300 times the minimally effective dose has been injected before stimulation of follicular growth occurred.

The enlarged ovaries and multiple large follicles observed in the ovaries of hypophysectomized rats injected with concentrates of pregnancy urine do not give a quantitative measure of the amount of follicle stimulating substance present. The amount is probably quite low, except perhaps during the early peak period. The amount of follicular development, and the extent of the ovarian weight increases are presumably amplified by the synergistic action with the interstitial cell stimulating component.

The report here of follicle stimulation caused by pregnancy urine is not the first time unusual biological effects have been attributed to the so-called chorionic hormone. Evans, Simpson, Austin and Ferguson, 1933; Evans, 1936; and Evans and Simpson, 1950, noted that high levels of concentrates of urine from pregnant women, or men with embryonal carcinomata, caused much larger ovarian weights in normal immature female rats, more seminal vesicle development in immature male rats, and more testicular development in pigeons than had usually been reported to result from injection of chorionic hormone.

SUMMARY

Quantitative and qualitative analyses (mouse uterine and hypophysectomized rat ovarian responses, respectively) have been made of the hormone content of urines from 16 cases of pregnancy.

The titer of gonadotrophin in the urine, measured by the response of the mouse uterus, rose slightly by the 7th to 18th day post ovulation, the daily excretion being between 90 and 100 M.U.U. (or I.U.).

After this time the amount of the hormone in the urine began to rise with extreme rapidity, reaching values of 400,000 M.U.U. or more by 35 days after ovulation.

This abrupt increase in output occurs with remarkable constancy in different patients, when increases are plotted in terms of the post-ovulatory period, thus eliminating the variability due to different lengths of menstrual cycle.

Tested by the response of the hypophysectomized immature female rat's ovaries, the urine from the earliest period of pregnancy caused only inter-

stitial tissue repair. This hormonal effect was first seen on the 12th day after ovulation (cycle day 26).

The urinary concentrates from specimens obtained after the 33rd day of pregnancy caused stimulation of follicular growth in addition to repair or hypertrophy of the interstitial cells.

Pregnancy urine from this period also stimulated the thyroids of the hypophysectomized test animals.

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NOTES AND COMMENTS

THE NEED FOR THE PRESENCE OF CALCIUM FOR THE STIMULATION *IN VITRO* OF RAT ADRENAL GLANDS BY ADRENOCORTICOTROPHIC HORMONE

Saffran, Grad and Bayliss (1952) have shown that adrenocorticotrophic hormone (ACTH) increases the steroid production of rat adrenals *in vitro*. The same has been found with pig and beef adrenal cortex slices by Haynes, Savard and Dorfman (1952, 1953). We have found that the presence of calcium in the incubation medium is essential for the stimulation by ACTH.

METHODS

Two rat adrenals were bisected and incubated at 37°C in 0.75 ml. of a Krebs-Ringer glucose medium in semi-micro Warburg vessels for one hour. This medium was discarded and the adrenals were incubated for a further two hours in fresh medium with or without added ACTH. The methods used by Saffran and Bayliss (1953) of extracting and measuring the steroids by the ultra violet absorption of the chloroform extractable lipid material have been modified so that 2–5 μg. steroid may be measured (Elliott *et al.*). In some of the experiments the steroids have been measured also by the use of 2,5 diphenyl-3-(4-styryl phenyl) tetrazolium chloride, M & B 1767 (Elliott *et al.*).

RESULTS

In Table 1 are given the results of experiments in which the effect of omitting calcium from the medium was tested. When Krebs-Ringer 0.01M phosphate was used for the incubation of the glands (20 to 50 mg. tissue/ml. medium), the pH of the medium at the end of the incubation was 6.7 to 6.9, although the medium with and without ACTH added had been adjusted to pH 7.4. The buffer in the medium was therefore increased to 0.03M and calcium was omitted since it precipitates to a variable extent at this phosphate concentration. With this latter medium no stimulation occurred. This is shown in the first values of Columns 5 and 6. The next data in Columns 5 and 6 show that with 0.01M phosphate also, ACTH has no effect on steroid production in the absence of calcium. To ensure that the low steroid values and lack of ACTH stimulation were not due simply to refractory glands instead of to absence of calcium, four pairs of experiments were carried out, the results of which are given in Columns 1 and 2. The steroid production was two to four times as great for the left adrenal pair to which calcium had been added along with ACTH as for the right pair that had received ACTH but no calcium. In fact these results are similar to the data in Columns 3 and 4 which illustrate the stimulation of steroid production by ACTH in the presence of calcium.

These experiments were repeated in Krebs-Ringer 0.025M bicarbonate medium, pH 7.4, because in this medium the pH can be maintained during the incubation and at the same time calcium can remain in solution. The same calcium effects as in the phosphate media were obtained. The results in Columns 7 and 8 show that calcium alone has no effect on the steroid production of the adrenal without added ACTH.

The steroid values obtained by use of the tetrazolium compound, although lower than the values obtained by the ultra violet method also showed the same calcium effects.

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TABLE 1. EFFECT OF CALCIUM ON STEROID PRODUCTION IN THE PRESENCE AND ABSENCE OF ACTH

Column No.	1	2	3	4	5	6	7	8
Ca 2.5 mM	+	−	+	+	−	−	+	−
ACTH	+	+	+	−	+	−	−	−
	L	R	L	R	L	R	L	R
	μg. steroid		μg. steroid		μg. steroid		μg. steroid	
Krebs-Ringer Phosphate	38 40 21 29	11* 13* 10 9	30	15	10* 7	9* 9		
Krebs-Ringer 0.025 M Bicarbonate-5% CO^2	29 *14*	14 *1*	58 *29*	22 *13*	12 *8*	14 *5*	21 *13*	24 *10*

* The steroid values were measured as cortisone, per 100 mg. adrenal tissue by the ultra violet method, those in italics by M & B 1767 on an aliquot of the solution for which the u.v. value is given. Because of animal variation each value in the L column (the steroid production of two left adrenals) may be compared only with the value to its right in the R column (the steroid production of the two right adrenals of the same animals). The phosphate concentration of the Krebs-Ringer phosphate medium was 0.01 M except in those experiments whose steroid values are marked with an asterisk in which it was 0.03 M. Connaught ACTH, Lot 8-3, was used. Its concentration was either 0.05 or 0.10 units/ml. medium for a given pair of experiments depending on the size of the glands. In experiments with phosphate media the amount of ACTH/100 mg. tissue was 0.18 to 0.24 units, in those with bicarbonate medium 0.13 to 0.17 units (Table 1) and 0.13 to 0.24 units (Table 2).

Further experiments were carried out to find whether the steroid production in the presence of ACTH would be affected by changes in the calcium concentration from 2.5 mM, the concentration equal to the average total calcium concentration in serum and that usually present in Krebs-Ringer medium. The results of these experiments are given in Table 2.

TABLE 2. EFFECT OF VARIOUS CALCIUM CONCENTRATIONS ON THE STIMULATION OF STEROID PRODUCTION BY ACTH

Column No.	1	2	3	4	5	6	7	8
Ca mM	2.5	10.0	0.5	0.1	0.5	0.5	0.1	0.1
ACTH	+	+	+	+	+	−	+	−
	L	R	R	R	L	R	L	R
	μg. steroid				μg steroid		μg. steroid	
Krebs-Ringer 0.025 M Bicarbonate 5%CO_2	57 43 36 *20* 45 *29*	56	41 36 *28*	20 *11*	29	10	25 *14* 13	15 *5* 12

Comparison of the values in Columns 2 and 3 with the appropriate values in Column 1 show that the calcium concentration can be varied from 0.5 to 10mM without affecting the steroid production of the glands in the presence of ACTH. However, in the presence of 0.1 mM calcium the steroid production with added ACTH is lower than in the presence of 2.5 mM calcium (Columns 4 and 1). In the presence of 0.5 mM calcium the steroid

production of glands incubated with ACTH was three times that of glands incubated without ACTH (Columns 5 and 6). In the presence of 0.1 mM calcium, the ACTH stimulation was unreliable (Columns 7 and 8).

SUMMARY AND DISCUSSION

The results of these experiments indicate that the presence of calcium in the medium is essential for the response of the adrenal to ACTH added *in vitro*, but that it has no effect on the steroid output in the absence of added ACTH. The mechanism of the calcium effect remains to be explained. It may be that calcium in the medium permits freer access of ACTH to the cellular mechanisms involved in steroid production, or that calcium is a cofactor in one of the reactions required for steroid formation.

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SAFFRAN. M., B. GRAD AND M. J. BAYLISS: *Endocrinology* **50:** 639. 1952.

THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday, and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 19, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the Sir Francis Drake Hotel, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. George W. Thorn, Peter Bent Brigham Hospital, Boston, Massachusetts, not later than February 1, 1954. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   Line 3. Institution of origin and city in which institution is located.
3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

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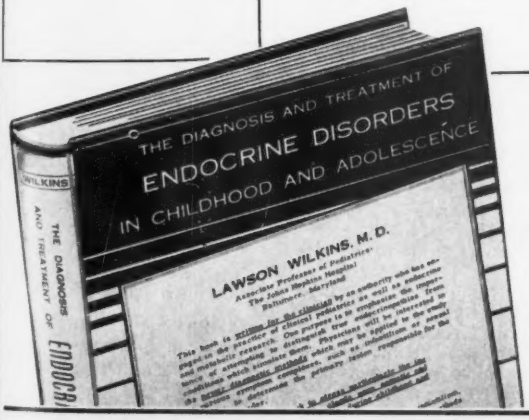

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